I hereby certify that this correspondence is being deposited with the United States Postal Service on the date set forth below as First Class Mail in an envelope addressed to: Commissioner for Patents, Box 1450, Arlington, A 22313-1450.

Date of Signature and Deposit: November 11, 2004

Sara D. Vinarov, Reg. No. 48,524

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Eric W. Triplett Thomas C. Herlache	Date: November 11, 2004
Serial No.:	09/927,616	Group Art Unit: 1638
Filed:	August 10, 2001	Examiner: Georgia L. Helmer
Title:	BIOLOGICAL CONTROL OF CROWN GALL DISEASE	File: 960296.97273

DECLARATION UNDER 37 CFR §1.132

Commissioner for Patents
P O Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Eric W. Triplett, on oath say and declare that:

1. I am the same Eric W. Triplett who is one of the named inventors of the above-identified patent application. I make this declaration in support of that patent application. I am currently employed by the University of Florida at the Institute of Food and Agricultural Sciences. I am a professor and the chair of the Department of Microbiology and Cell Sciences. I have worked as a research scientist specializing in the general area of plant-microbe interactions for 26 years. I have published extensively in this area. A copy of my Curriculum Vitae is attached as Exhibit A.

2. I have reviewed the Office Actions issued in this matter by the U.S. Patent and Trademark Office on February 18, 2004 and on August 12, 2004. I understand that currently Claims 1-4, 6, 13, 15-22, 24 and 25 are rejected for an alleged lack of enablement. It appears that the Examiner was not convinced that the method and composition for biologically controlling crown gall disease would be applicable to taxonomically diverse species of plants. Accordingly, this Declaration is submitted to provide evidence that indeed all plant species susceptible to crown gall disease are capable of being biologically controlled via a TFX-producing strain of α-proteobacteria, such as *Rhizobium and Agrobacterium*.

3. At my direction, and under my supervision, Yuemei Dong, a postdoc formerly in my laboratory has demonstrated in the manner described in the application that trifolitoxin-producing bacteria was also able to prevent crown gall disease on grapes, as was predicted in the patent application. Indeed, one would agree that clearly, grapes are taxonomically diverse from *Nicotiana glauca.*

4. I note that recent laboratory experiments conducted by my research group have also shown that trifolitoxin-producing bacteria prevented crown gall disease on two cultivars of grapes. The results of these experiments have been summarized in Table 1 herein below. Copies of photographic images illustrating control or inhibition of crown gall disease on a variety of grapes are also enclosed herewith as Exhibits B-G.

Table 1 shows that a non-recombinant trifolitoxin-producing strain, *Rhizobium leguminosarum* bv. trifolii T24, can reduce crown gall formation. Therefore, the results shown in Table 1 further illustrate that: 1) trifolitoxin production in three different *Rhizobiaceae* backgrounds (*R. leguminosarum* strain CE3, *R. etli* strain T24 *and A. vitis* strain F2/5) are capable of controlling crown gall; and 2) the efficacy of the methods of the invention goes well beyond simply *Nicotiana glauca* to any plant species susceptible to crown gall disease.

Table 1

	Phenotype of co-inoculum	Candice CG78	Concord CG78	Concord CG435
Water	Control	0	0	0
F2/5(pT2TFXK)	TFX+	100	83	ND
F2/5(pT2TX3K)	TFX-	0	0	ND
CE3(pT2TFXK)	TFX+	ND	ND	83
CE3(pT2TX3K)	TFX-	ND	ND	0
T24	TFX+	ND	ND	50
T24::Tn5-1	TFX-	ND	ND	0

Specifically, Table 1 shows percent control of crown gall disease on two lines of grape plants (Candice or Concord) following inoculation with either of two virulent stains of *Agrobacterium vitis* (CG78 or CG435) by co-inoculation with a strain producing TFX (TFX+) or a non-producing strain (TFX-). I note that ND refers to the phrase not determined. Each data point represents at least eight replicates. These results show that plants beyond *Nicotiana glauca* are susceptible to biological control of crown gall disease via a TFX-producing strain of α-proteobacteria.

5. Furthermore, I would like to emphasize that it is well recognized within the plant pathology art that *N. glauca* is a suitable indicator plant for crown gall assays. (See *Annual*

Review of Phytopathology, September (1999), vol. 37, pp. 53-80, specifically the section on "Genes Associated With Host-Pathogen Interactions".) A copy of this reference is enclosed herewith as Exhibit H. I specifically note that the operative idea in the experiments described in the present application is that galling on *N. glauca* is representative of galling of plants in general, because *N. glauca* is well known in this art as an indicator plant.

6. I believe that crown gall can be controlled in all plant species susceptible to the disease. Susceptible plants are also well-known in the art, see for example: http://www.britannica.com/ebc/article?tocId=9361941 (date viewed online, September 30, 2004); where it recites that "thousands of plant species are susceptible, including especially rose, grape, pome and stone fruits (e.g., apples, peaches), shade and nut trees, many shrubs and vines, and perennial garden plants."

Furthermore, it is now also well known to those of ordinary skill in the art that a specific group of α-proteobacteria (i.e., *Ochrobactrum, Rhodobacter, Rhodopseudomonas, Brucella, Rhizobium and Agrobacterium,* not *Bradyrhizobium*) is capable of being inhibited by trifolitoxin. The ability of our method and crown gall biocontrol agents to control galling on both *N. glauca* and *Vitis* spp. demonstrates that taxonomically divergent species of plants have been found that are responsive to the crown gall controlling α-proteobacteria. These results also demonstrate the efficacy of using the methods and compositions of the invention to control crown gall beyond simply *Nicotiana glauca.*

7. More specifically, it is now well known that *Agrobacterium* causes crown gall on over 200 different plants, including relatives of cranberry (*e.g.*, blueberry and rhododendron), along with dicots and other woody plants such as grape, raspberry, and nut trees. These plants may all display crown gall disease when the pathogenic bacterium, such as for example, *Agrobacterium tumefaciens* carrying a pTi plasmid, enters the plant through a wound site. The bacteria then cause gall formation at the wound site. Infections frequently occur on the crown of the plant, which is the tissue near the junction of the root and stem and is generally near the soil surface. These infections give rise to the common name for the disease, i.e., crown gall. In some cases, pathogenic bacteria may systemically colonize a plant. For example, *A. vitis* is known to systemically colonize grapevines. Wounds that occur far from the soil, e. g., on canes or vine trunks, may display crown gall disease after wounding of the canes. Such wounds may occur for example, during regular vineyard pruning, or by frost injury, thereby rendering the plants susceptible to crown gall disease.

8. Furthermore, I believe it is valuable to note that nodulation and galling are very different processes, and more importantly, occur in very different environments. In

particular, rhizobia infect root hairs and Agrobacterium infects wounded or de-differentiated (e.g., callus culture) plant tissue. In a wound, the infecting bacteria are exposed to all sorts of things that have leaked out of the plant cell, including proteases that could deactivate the TFX peptide. In fact, the deactivation of peptide antibiotics has appeared to be a problem with early attempts at obtaining disease control by expressing bacteriolytic peptides in transgenic plants. (See Herlache TC, and Triplett EW, "Expression of a crown gall biological control phenotype in an avirulent strain of Agrobacterium vitis by addition of the trifolitoxin production and resistance genes" *BMC Biotechnology* 2002, 2:2.)

9. Also, generally speaking, *in vitro* antibiotic activity is neither necessary nor sufficient for biological control in plants. (See for example Burr, T. J., et al., "Biological control of grape crown gall by strain F2/5 is not associated with agrocin production or competition for attachment sites on grape cells Phytopathology" 1997; 87(7): 705-711.) This reference shows that *in vitro* antibiosis is not necessary for biocontrol. Many antibiotic producing strains have been found to be ineffective biocontrol agents. Unfortunately, I have found that most of this negative data has not been published. However, note that in Samac, D.A., et al., "Effects of antibiotic-producing Streptomyces on nodulation and leaf spot in alfalfa" *Applied-Soil-Ecology*. 2003; 22(1): 55-66, only two of fifteen *in vitro* antibiotic producing strains controlled disease in seed inoculation assays. Also see, Schmiedeknecht, G., et al. "Use of *Bacillus subtilis* as biocontrol agent. V. Biological control of diseases on maize and sunflowers" *Zeitschrift-fuer-Pflanzenkrankheiten-und-Pflanzenschutz*. 2001; 108(5): 500-512; where biocontrol did not correlate 1:1 with *in vitro* antibiotic production. I note that these are only a few of the many references which illustrate that *in vitro* antibiosis is not sufficient for biocontrol.

10. I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further, that these statements are made with knowledge that willful false statements, and the like so made, are punishable by fine or imprisonment, or both, under Section 1001, Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Further declarant sayeth not.

Eric W. Triplett

Dated: November 11, 2004

Eric W. Triplett

Eric W. Triplett, Professor and Chair

Department of Microbiology and Cell Science
University of Florida
1052 Museum Road
Gainesville, FL 32611
1-352-392-1906 (phone)
1-352-392-5922 (fax)
email: ewt@iufl.edu

Date/Place of Birth: July 11, 1954, Philadelphia, Pennsylvania
Citizenship: U.S.A.

EDUCATION:

1976 B.S., Biology, Cook College, Rutgers University, New Brunswick, New Jersey
1978 M.S. Botany/Plant Physiology, University of Maryland, College Park, Maryland
1981 Ph.D. Agronomy/Plant Physiology, University of Missouri, Columbia, Missouri

PROFESSIONAL CAREER:

1976 Laboratory Assistant, Entomology Department, Rutgers University, New Brunswick, New Jersey, Dr. E.J. Hansens, Supervisor, toxicology work on house flies.

1976-1978 Graduate Teaching Assistant, Botany Department, University of Maryland, College Park, Maryland, Taught botany and plant physiology labs, Drs. Blevins and Curtis, supervisors.

1978-1981 Graduate Research Assistant, Agronomy Department, University of Missouri, Columbia, Missouri, Dr. D.G. Blevins, Advisor, Nitrogen metabolism in soybeans.

1981-1982 Postdoctorate, Biochemistry Department, University of Wisconsin-Madison, Dr. P.W. Ludden, Supervisor, Nitrogenase regulation in *Rhodospirillum rubrum.*

1982-1987 Assistant Professor, Plant Pathology Department, University of California, Riverside, Nitrogen fixation research

1987-1990 Assistant Professor, Agronomy Department, University of Wisconsin-Madison

1990-1995 Associate Professor, Agronomy Department, University of Wisconsin-Madison

1995-2003 Professor, Agronomy Department, University of Wisconsin-Madison
Research in molecular microbial ecology, plant-microbe interactions

EXHIBIT A

2003 Fulbright Award, visiting scholar at the Research School of Biological Sciences, Australian National University, Canberra, January- July, 2003.

2003-present Professor and Chair, Microbiology and Cell Science Department, University of Florida, Gainesville

PROFESSIONAL SOCIETIES:

American Association for the Advancement of Science
American Phytopathological Society
American Society for Microbiology
International Society for Molecular Plant-Microbe Interactions
American Society of Plant Biologists
Editorial Board Member of Applied and Environmental Microbiology (1997-2003)

INVITED PRESENTATIONS (last five years):

Invited speaker, 16th North American Symbiotic Nitrogen Fixation Conference, Cancun, Mexico, February 5, 1998
Seminar, Novartis, Research Triangle Park, North Carolina, February 17, 1998
Invited Speaker, 11th International *Trifolium* Conference, Madison, WI, June 11, 1998
Invited Speaker, Consortium for Plant Biotechnology Research, Washington, DC, March 3, 1999
Invited Speaker, APS/CPS Annual Meeting, Montreal, Canada, August 1999
Invited Speaker, 12th International Congress on Nitrogen Fixation, Foz do Iquaçu, Brazil, September 14, 1999
Seminar, University of Georgia, Athens, December 9, 1999
Invited Speaker, Agricultural Microbes I Conference, San Diego, CA, January 14, 2000
Invited Speaker, 17th North American Conference on Symbiotic Nitrogen Fixation, University of Laval, Sainte Foy, Quebec, Canada, July 28, 2000
Invited Speaker, 8th International Symposium on Nitrogen Fixation with Non-Legumes, University of Sydney, Australia, December 5, 2000.
Seminar, University of California, Davis, February 2, 2001.
Invited Speaker, 13th International Congress on Nitrogen Fixation, Hamilton, Ontario, July 6, 2001.
Seminar, Iowa State University, Ames, Iowa, September 27, 2001.
Seminar, Ohio State University, March 14, 2002
Invited Speaker, 102nd Annual Meeting of the American Society for Microbiology Salt lake City, UT, May 22, 2002.
Invited Speaker, 18th North American Conference on Symbiotic Nitrogen Fixation, Columbia, MO, June 5, 2002.
Invited Speaker, 9th International Symposium on Nitrogen Fixation in Non-legumes, Leuven, Belgium, September 5, 2002.
Seminar, The Australian National University, National Center for Epidemiology and Public Health, Canberra, June 5, 2003.
Invited Speaker, 11th International Congress on Molecular Plant-Microbe Interactions, St. Petersburg, Russia, July 20, 2003.
Invited Speaker, 12th Plant & Animal Genome Conference, San Diego, January 11, 2004.

Invited Speaker, 19th North American Symbiotic Nitrogen Fixation Conference, Bozeman, MT, July 1, 2004.

Invited Speaker, 10th International Symposium on Microbial Ecology, Cancun, Mexico, August 2004.

Current support:

NSF, A microbial observatory for the North Temperate Lakes Long Term Ecological Research Site, $1,000,000, 11/10/99-10/31/04, E.W. Triplett (lead PI), with co-PIs L.K. Graham, S.R. Carpenter, T.K. Kratz, and D.E. Armstrong

NSF, A comparative study of a suite of lakes in Wisconsin, 11/01/02-10/31/08, S.R. Carpenter (lead PI), E.W. Triplett one of 26 co-PIs. (supports 0.5 graduate research assistant in Triplett laboratory).

Pending support:

NSF/USDA, Genome sequencing of a nitrogen-fixing bacterial endophyte, B.A. Methe, D.E. Fouts, and E.W. Triplett, $770,148 requested.

USDA, Analysis of a nitrogen-fixing association between wheat and *Klebsiella pneumoniae* 342, E.W. Triplett, F. Altpeter, $500,000 requested.

PATENTS (all owned by the Wisconsin Alumni Research Foundation):

"Recombinant *Rhizobium* bacteria inoculants", issued February 2, 1993, no. 5,183,759
"Plasmid for transformation of root nodule bacteria", issued January 12, 1999, no. 5,858,762.
"Enhanced inoculant for soybean cultivation", issued May 25, 1999, no. 5,906,929.
"Novel microorganisms", issued June 1, 1999, no. 5,908,758.

PUBLICATIONS:

Scupham, A.J. and E.W. Triplett. 2004. Determination of the amino acid residues required for the activity of the anti-rhizobial peptide antibiotic trifolitoxin. Submitted to Molecular Plant-Microbe Interactions (received 6/16/04).

Iniguez, A.L., Y. Dong., H.D. Carter, B.M.M. Ahmer, J.M. Stone, and E.W. Triplett. 2004. Regulation of enteric endophytic bacterial colonization by plant defenses. Submitted to Molecular Plant-Microbe Interactions, (in press).

Yannarell, A.C. and E.W. Triplett. 2004. Geographic and environmental sources of variation in lake bacterial community composition. Applied and Environmental Microbiology (in press).

66. Iniguez, A.L., Y. Dong, and E.W. Triplett. 2004. Nitrogen fixation in wheat provided by

Klebsiella pneumoniae 342. Molec. Plant-Microbe Interact. 17:1078-1085.

65. Graham, J.M., A.D. Kent, G.H. Lauster, A.C. Yannarell, L.E. Graham, T.K. Kratz, and E.W. Triplett. 2004. Seasonal dynamics of phytoplankton and protoplankton communities in a northern temperate humic lake: diversity in a dinoflagellate dominated system. Microbial Ecology (in press).

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63. Iniguez, A.L., E.A. Robleto, A.D. Kent, and E.W. Triplett. 2004. Significant yield increase in *Phaseolus vulgaris* obtained by inoculation with a trifolitoxin-producing, Hup^+ strain of *Rhizobium leguminosarum* bv. phaseoli. Crop Management (online only: http://www.plantmanagementnetwork.org/pub/cm/review/2004/yield/, 5 pages).

62. Yannarell, A.C. and E.W. Triplett. 2004. Within- and between-lake variability in the composition of bacterioplankton communities: investigations at multiple spatial scales. Appl. Environ. Microbiol. 70:214-223.

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60. Kent, A.D., D.J. Smith, B.J. Benson, and E.W. Triplett. 2003. Web-based phylogenetic assignment tool for analysis of terminal restriction fragment length polymorphism profiles of microbial communities. Appl. Environ. Microbiol. 69:6768-6776.

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53. Kent, A.D. and E.W. Triplett. 2002. Microbial communities and their interactions in soil and rhizosphere ecosystems. Annu. Rev. Microbiol. 56:211-236.

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Prokaryotic Nitrogen Fixation: a Model System for the Analysis of a Biological Process, E.W. Triplett, ed., Horizon Scientific Press, Norfolk, UK, pp. 779-792.

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29. Borneman, J. and E.W. Triplett. 1997. Molecular microbial diversity in soils from eastern Amazonia: evidence for unusual microorganisms and population shifts associated with deforestation. Appl. Environ. Microbiol. 63:2647-2653.

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35. Palus, J.A., J. Borneman, P.W. Ludden, and E.W. Triplett. 1996. Isolation and characterization of endophytic diazotrophs from *Zea mays* L. and *Zea luxurians* Iltis and Doebley. Plant and Soil. 186:135-142.

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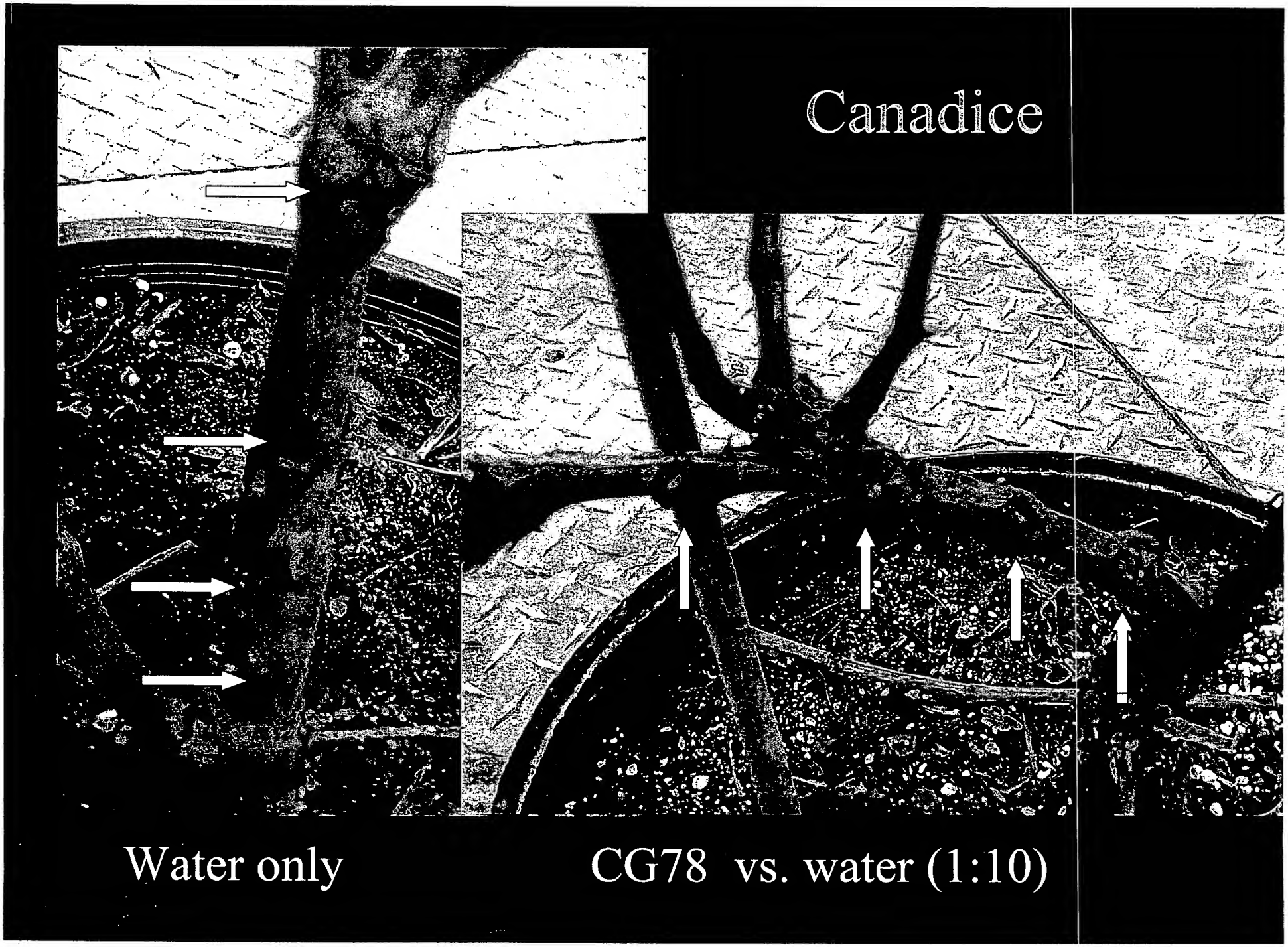
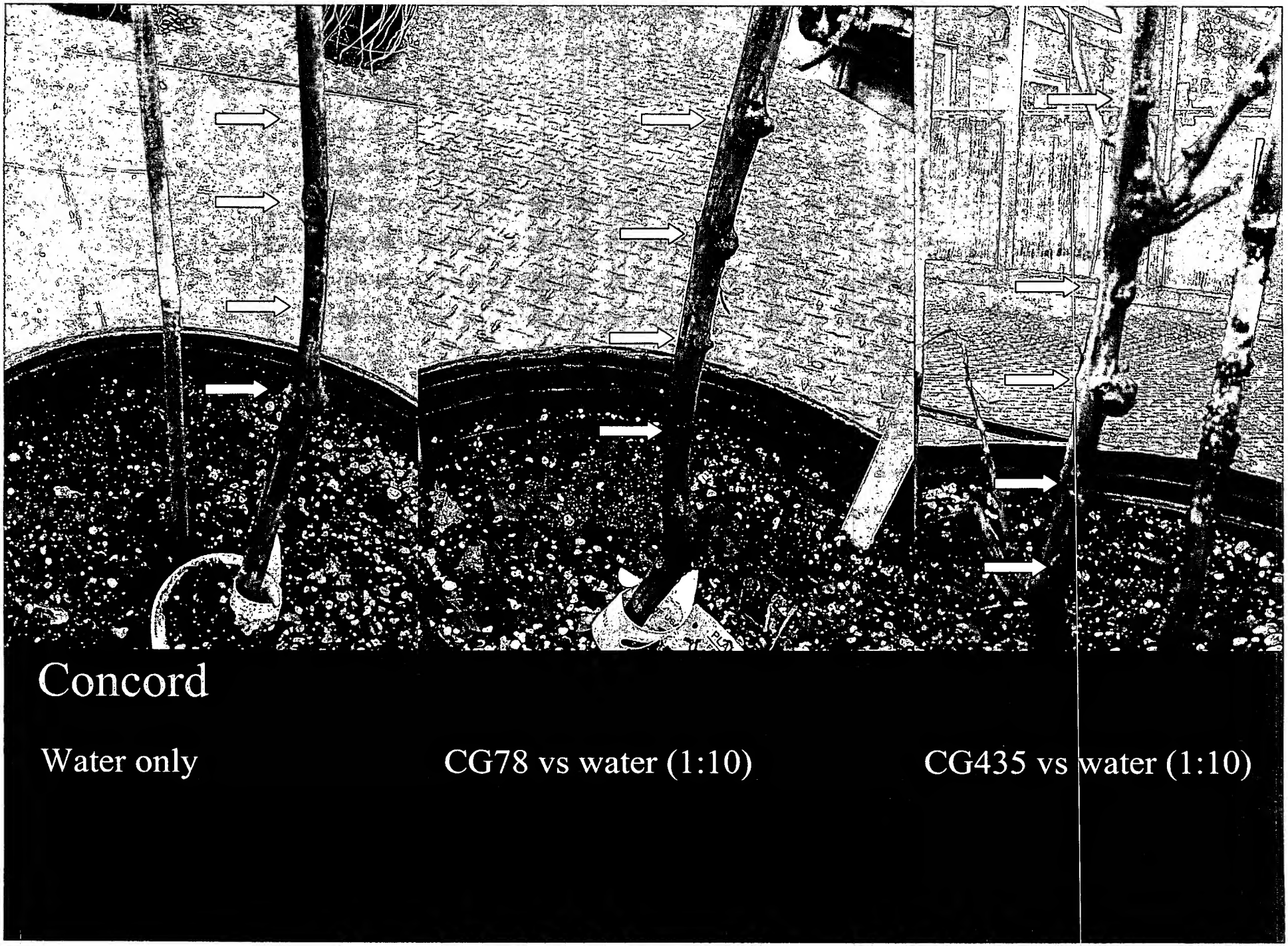


Exhibit B

Exhibit C



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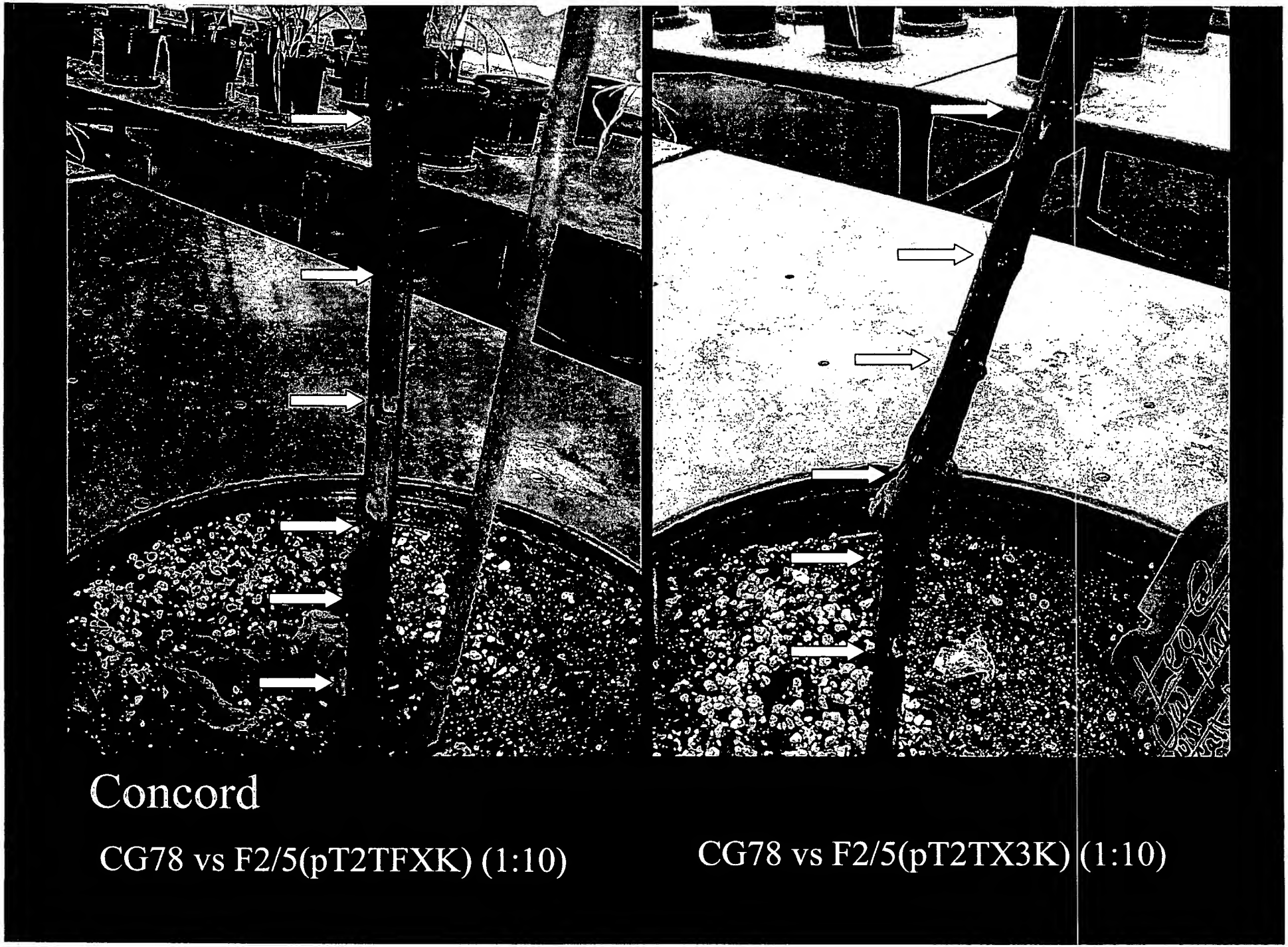

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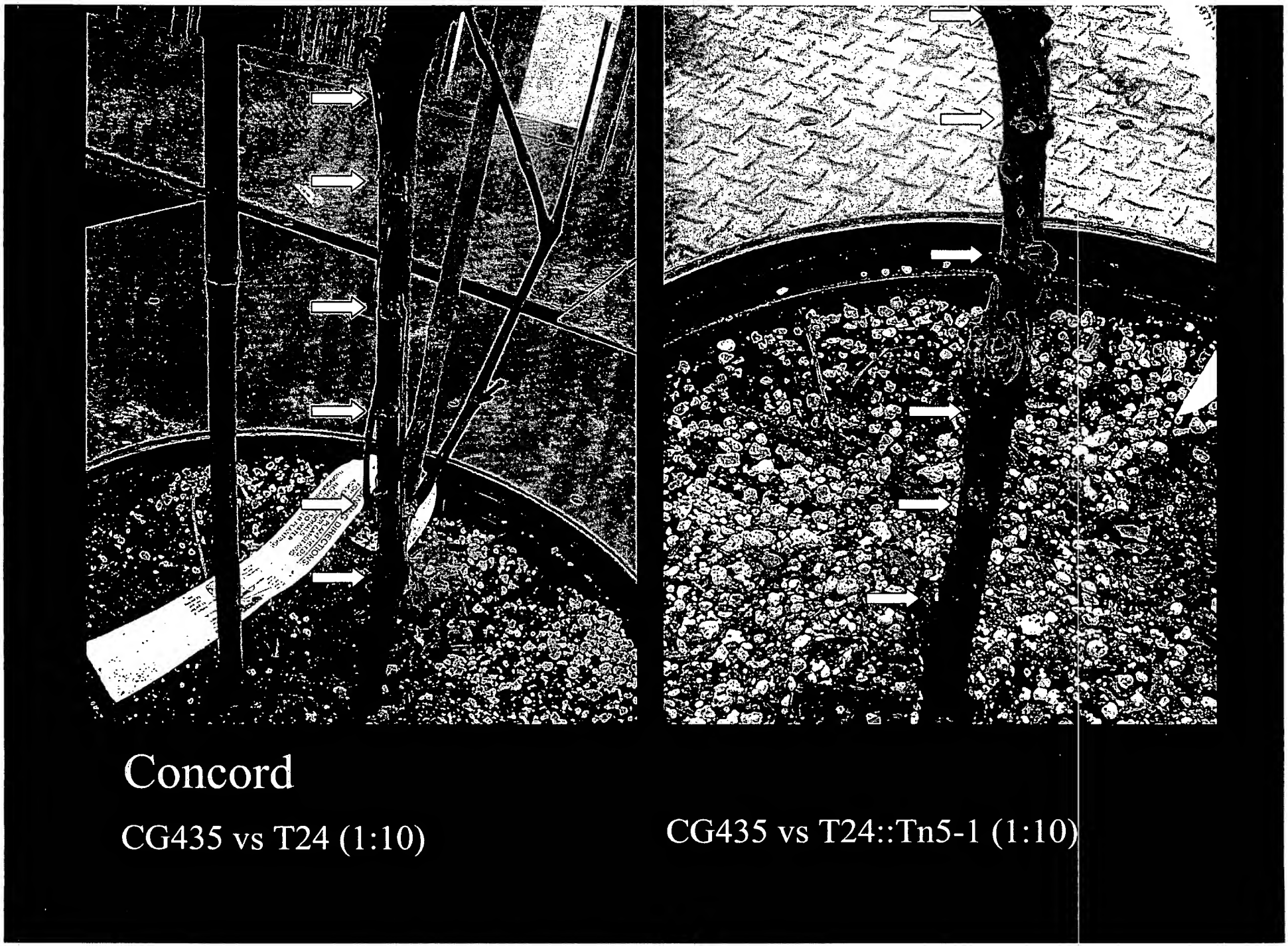

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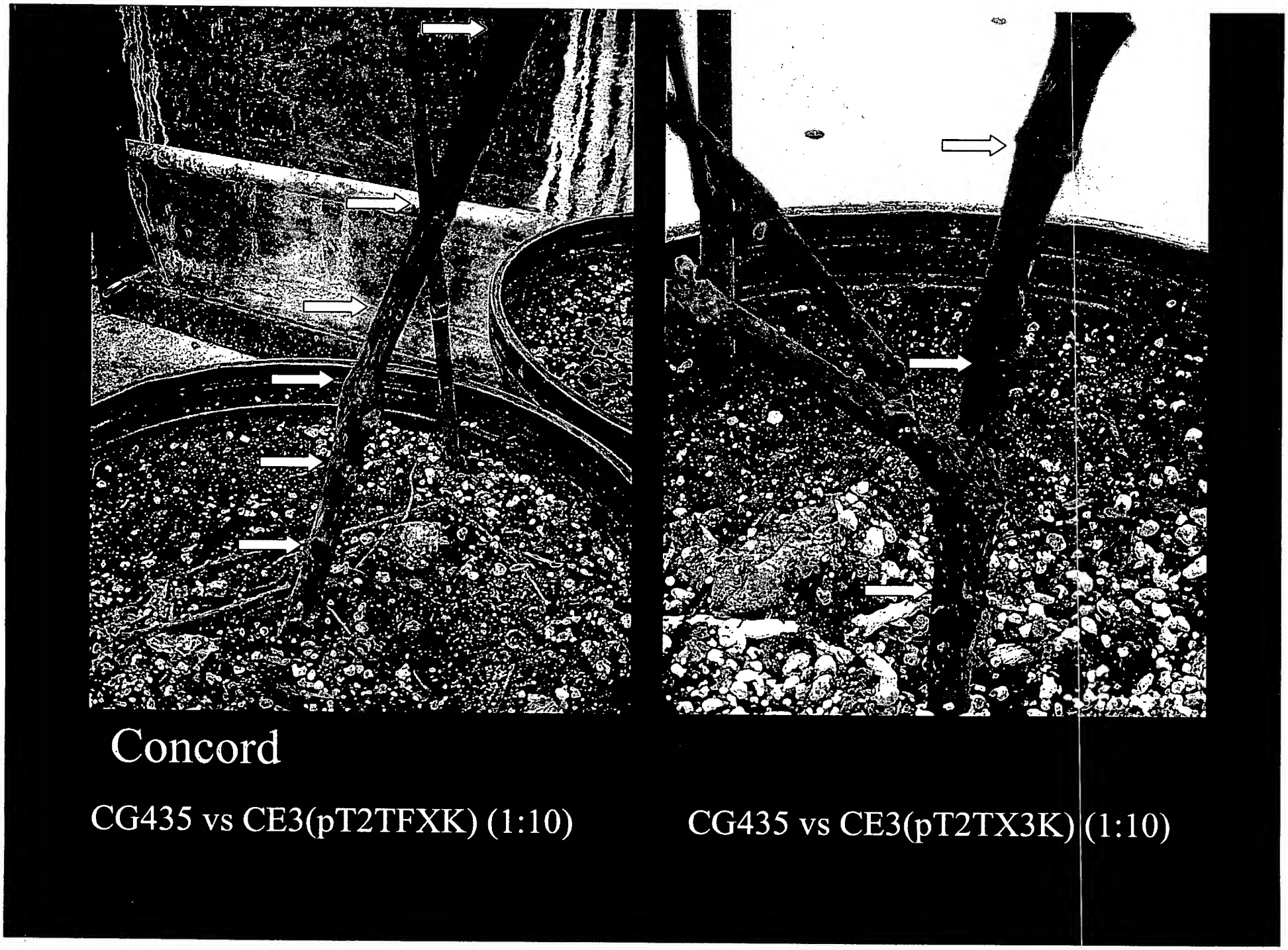

Concord

CG435 vs CE3(pT2TFXK) (1:10)

CG435 vs CE3(pT2TX3K) (1:10)

Exhibit G

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CROWN GALL OF GRAPE: Biology and Disease Management

Thomas J. Burr and Leon Otten
Department of Plant Pathology, New York State Agricultural Experiment Station, Cornell University, Geneva, NY 14456 and Institute of Plant Molecular Biology, 12 rue du General Zimmer, 67084 Strasbourg, France; e-mail: tjb1@cornell.edu

Key Words *Agrobacterium vitis*, evolution, genetic diversity, plasmids, taxonomy

■ **Abstract** Not until 1973 was it reported that strains of Agrobacterium that cause crown gall disease of grape form a specific group (later characterized as *Agrobacterium vitis*). Tumorigenic and nontumorigenic *A. vitis* have since been isolated from infected and symptomless grapes worldwide. Research on the genetic makeup of *A. vitis* has led to an improved understanding of pathogen biology and bacterial evolution. In addition, the identification of significant gene sequences has facilitated the development of PCR and RFLP-based identification procedures that continue to improve the detection of *A. vitis* in plants and soil. Current control practices rely on the use of disease-resistant cultivars, cultural practices that minimize plant injury, and the production of pathogen-free vines. Promising future controls include employment of biological control agents and development of crown gall–resistant transgenic grapevines.

INTRODUCTION

Crown gall has been recognized as a plant disease of worldwide importance on many plant species for more than 100 years (28). A discovery by Braun & Mandle (9) in 1948 provided major insight into the crown gall infection process and paved the way for intensive research on the molecular biology of infection: Once plants were infected by *Agrobacterium tumefaciens*, the tumor tissue could be cultured on a defined, hormone-free medium in the absence of the bacterium. Thus, something of unknown nature [the tumor-inducing factor (TIP)] was being transferred by the bacterium to the plant cell. Almost 30 years later, it was demonstrated that the genes associated with tumor formation are carried on a plasmid (Ti plasmid). Major discoveries included the following: *A. tumefaciens* transfers tumorigenicity factors from tumorigenic to nontumorigenic strains (52); the presence of the Ti plasmid in the bacterium is positively correlated with tumorigenicity (111); and bacterial DNA from the Ti plasmid (the transfer of T-DNA) is integrated into the plant genome (29). Ti plasmids also carry genes that are involved in synthesis and catabolism of a diverse group of small molecules called opines. Opines are synthesized in

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and secreted from galls. The rather unique ability of Agrobacterium to catabolize them gives the bacterium a competitive advantage in the vaccinity of galls. Today, highly active research programs continue to study the mechanisms by which Agrobacterium transfers Ti plasmids between bacteria and T-DNA into plants.

Some of the earliest reports of crown gall were of the disease on grape (41). It was repeatedly observed that injuries on grape trunks caused by freezing temperatures provide common sites for crown gall infections and that grape cultivars vary in their susceptibility to the disease. The widespread occurrence of the disease and variation in cultivar susceptibility are reflected in results of a multicountry crown gall survey reported by the Office International de la Vigne et du Vin, which was recently summarized (14). The impact of crown gall on vine growth and yield was assessed in a four-year study in California on the cultivar Zinfandel (86). Vines with 50% or greater of their trunk circumference affected with crown gall had significantly less growth than mildly diseased vines had. Crown gall may also cause significant losses in nurseries by affecting vine growth and necessitating the destruction of infected plants.

AGROBACTERIUM VITIS

Although tumorigenic *Agrobacterium* spp. were isolated from grape galls for many years, strains from grape were first compared with those from other plants by Panagopoulos & Psallidas in 1973 (72). By comparing responses to various biochemical and physiological tests, they concluded that strains from grape formed a distinct group and that their tumorigenicity was often host-limited. Kerr & Panagopoulos (53) and Süle (92) later proposed that strains from grape be classified as biovar 3 (or biotype 3) of *A. tumefaciens*. In the following years, it became apparent that the vast majority of strains isolated from crown gall–diseased grapevines worldwide belonged to biovar 3 and that there are wide host range (WHR) and limited host range (LHR) strains. In addition to having their physiological and biochemical traits, biovar 3 strains were insensitive to agrocin 84 in vitro, and their tumorigenicity on grape was not suppressed by *A. radiobacter* strain K84. Biovar 3 strains were also differentiated from other biovars by reaction to a monoclonal antibody (4) and by their ability to induce a grape-specific necrosis (16).

Further taxonomic evaluation of biovar 3 strains by Ophel & Kerr in 1990 resulted in the naming of new species, *A. vitis* (62). In addition to the criteria listed above, they compared DNA homology between *A. vitis* strains and other *Agrobacterium* spp. More recently, several other methods have been used to characterize *A. vitis*. Jarvis et al (48) used fatty acid analysis to identify species of 65 strains of *Agrobacterium* spp. and 150 strains of *Rhizobium* and *Sinorhizobium*. A two-dimensional plot analysis of fatty acid compositions revealed that *A. vitis* is more similar to *Rhizobium galegae* than it is to other *A. tumefaciens* or *A. rhizogenes*. Bouzar & Jones (8) also showed that *A. vitis* can be differentiated from other *Agrobacterium* spp. by fatty acid analysis. It was determined that

cis-vaccenic acid is predominant in all Agrobacterium strains, whereas the level of 3-hydroxypalmitic acid differs among species: *A. vitis* produces significantly less than *A. tumefaciens* or *A. rhizogenes*. Interestingly, *A. vitis* produces the highest detectable level of 10 methylnonadecenoic acid, and only *A. vitis* and *A. rhizogenes* produce detectable levels of arachidonic acid.

A. vitis was also differentiated from other *Agrobacterium* spp. by using the GN Microplate system (BioLog, Inc.). They form a distinct group based on utilization of 95 different carbon sources. Major differences between *A. vitis* and all other species tested were that relatively few *A. vitis* strains utilized D-galactonic acid lactone, whereas the majority of strains utilized *p*-hydroxyphenylacetic acid. With BioLog and fatty acid analysis, species of Agrobacterium formed distinct clusters regardless of tumorigenicity.

In addition to fatty acid analysis, Weibgen et al (107) investigated the lipopolysaccharide (LPS) composition of nine Agrobacterium strains (including three *A. vitis* strains) and determined that *A. vitis* and *A rubi* contain an R-type LPS, whereas *A. tumefaciens* and *A. rhizogenes* have very long O-chains with more than 20 O-repeating subunits. The rare sugar derivative 3-deoxy-*lyxo*-heptulosaric acid was found at trace amounts in all species but at highest levels in all LPS fractions of *A. vitis*. An *A. vitis*–specific monoclonal antibody did not react with the isolated LPS from the bacterium.

Comparisons of *A. vitis* with other related species were also made at the DNA level. Sawada compared DNA sequences of a short variable region (nucleotides 1019–1200) of the 16S rRNA gene (84). In this analysis, *A. vitis* was found to be most similar to *A. rubi* (only 2 nucleotide differences) and most dissimilar to *A. tumefaciens* (17 nucleotide differences). In a related study, 16S rRNA sequences from several genera in Rhizobiaceae were compared (108). The single *A. vitis* strain analyzed was 94–96% similar to *A. rhizogenes*, *A. rubi*, and *A. tumefaciens* but even more similar to a strain of *Rhizobium galegae* (same conclusion as for fatty acid analysis discussed above). More detailed and informative comparisons of entire *A. vitis* rRNA sequences are discussed later in this review.

Irelan & Meredith, using RAPDs, detected a high degree of inter- and intraspecific genetic diversity in total genomic DNA from a collection of Agrobacterium strains that included *A. vitis* (46). Four random primers were found to be most effective for detecting diversity, and *A. vitis* strains (all from California) were more diverse than other species, with only 12% of the PCR products being shared between strains. It was significant that the different species of *Agrobacterium* clustered separately according to RAPD patterns.

GENETIC DIVERSITY

The genetic diversity of *A. vitis* has been studied more thoroughly than for other *Agrobacterium* spp., probably because grape is a high-value crop and significant outbreaks of crown gall occurred in the United States and Europe after unusually

cold winters in the mid-1980s. Because of its phytopathological significance, many strains are available from various grape-growing areas in the world including Germany, France, Australia, Hungary, South Africa, and the United States. As is the case for *A. tumefaciens* and *A. rhizogenes*, most known pathogenic properties of *A. vitis* are encoded by genes on the Ti plasmid. Regions of the *A. vitis* genome, particularly the Ti plasmids, have been studied extensively. They show considerable diversity, which makes it possible to define genetic relationships between various strains, to identify representatives of the different groups, and to establish the phytopathological properties of model strains. It has also been possible to develop efficient identification and characterization methods for large numbers of strains.

Ti Plasmid Structure

Restriction maps, cloned fragments, and sequences are now available for the major types of *A. vitis* Ti plasmids. References to sequences of *A. vitis* strains (now adding up to about 110 kb) are listed in Table 1. The following Ti plasmids have been cloned and mapped:

1. Three O/C type Ti plasmids that have large TA-regions (O/C refers to genes that encode the synthesis of the opines octopine and cucumopine. TA refers to one of the two T-DNAs on the plasmids): pTiTm4 (256 kb) (69), pTiHm1 (258 kb) (104), and pTi2608 (248 kb) (37).
2. Two O/C type Ti plasmids that have small TA-regions: pTiAB3 (234 kb) (104) and pTiAg57 (224 kb) (104).
3. A nopaline type Ti plasmid, pTiAB4 of 157 kb (65), that is highly conserved in different *A. vitis* nopaline strains.

O/C large TA, O/C small TA, and nopaline type plasmids have identical virulence regions (65, 104) but differ in other regions. The ancestral Ti plasmid type has not yet been determined but the characteristic mixture of common DNA and specific DNA in the plasmids is a strong indication for plasmid recombination. Interestingly, the nucleotide sequences of common regions are more than 99.9% homologous, which suggests that the evolution of plasmids proceeds mainly by large-scale events like insertions and deletions (65, 71, 104). In one case, it was shown that a direct 2.3-kb repeat present in various O/C Ti plasmids can lead to the loss of a 66-kb fragment containing the TB-region (37). Large-scale events like deletions or insertions complicate attempts to construct phylogenetic trees. For example, we have shown that the genetic relationships between the Ti plasmids cannot be analyzed with RFLP-based techniques but only by careful analysis of Ti plasmid maps (104). In addition, repeated clonal expansion of only a few Ti types has led to a lack of evolutionary intermediates, which makes it even more difficult to reconstruct the evolutionary history of these structures. Such reconstructions are not only interesting from a theoretical point of view but are important for

TABLE 1 DNA that has been sequenced from *Agrobacterium vitis* as of December 1998

Strain (Ti type)	Description	Size (nt)	Accession no.
Chromosome			
K305 (O/C)	Repeated sequence, partial	132	S68163
CG49 (N)	*pehA*	2866	U73161
K309 (O/C)	*rrnA* operon	7276	U45329
S4 (V)	*rrnA* operon	7534	U28505
Ti plasmid			
CG474 (O)	Complete T-DNA	14960	U83986
AB4 (N)	*iaaM* to *nos* gene	6482	X77327
Tm4 (O/C)	Complete T-DNA	15463	U83987
Ag162 (O/C)	*iaaH*	1835	AF039169
AB3 (O/C)	IS*869*	863	X53945
AB3 (O/C)	IS*868*	1362	X55075
AB3 (O/C)	TA, *ocs*, partial	200	M63058
AB3 (O/C)	TA, right border	307	M63056
AB3 (O/C)	*acs*, partial	424	M91188
Ag57 (O/C)	TA, right border	185	M63057
Ag57 (O/C)	IS*870*	1754	Z18270
2608 (O/C)	RSAv-1 repeat	2563	Z22732
Ag162 (O/C)	*virA*	2730	X05241
Ag57 (O/C)	*virF*	922	AF044200
S4 (V)	T1-DNA	3720	M91608
S4 (V)	T2-DNA	5516	M91609
S4 (V)	T3-DNA	3271	M91610
TAR region			
AB4 (N)	*ttuA-ttuE*	7163	U25634
AB4 (N)	*ttuC*	1523	AF010261
AB4 (N)	ORFZ2	693	AF010414
pTiAB3 (O/C)	*ttuA*	227	AF010413
pTiAB3 (O/C)	*ttuC*	3405	AF010262
pTiAB3 (O/C)	*ttuC*	3172	AF010263
pTiAB3 (O/C)	ORFZ2	1041	AF010415
pTiAB3 (O/C)	*ttuA*-ORFZ2	11928	U32375

establishing the relatedness of the different plasmid forms and predicting their oncogenic properties and for predicting their capacity to evolve.

4. Many regions of the Ti plasmid from the vitopine strain S4 (262 kb) (39) are homologous to fragments of other Ti plasmids, but its origin of replication belongs to a new incompatibility group called IncRh-4 (96), and its virulence genes are arranged differently from other Ti plasmids.

Chromosome Structure

As mentioned previously, strains from grape were grouped together as biovar 3 strains of *A. tumefaciens* until further molecular and classical microbiological analysis led to the naming of a separate species, *A. vitis*. These studies have been confirmed and extended by sequence analysis of ribosomal RNA sequences. *A. vitis* strains contain four ribosomal RNA operons, *rrnA–D* (66, 67). The *Agrobacterium rrn* operons are about 7 kb in size and show conserved and variable regions. The variable regions can be used to construct very detailed intraspecific phylogenetic trees, whereas the conserved regions can be used to define relationships at higher taxonomic levels, for example, in comparisons with other genera like *Rhizobium* and *Bradyrhizobium*. Sequence studies on the *rrn* genes from the Rhizobiaceae family have shown that agrobacteria and rhizobia are not well-defined groups. *A. vitis*, for example, is more closely related to *R. galegae* than to other *Agrobacterium* species, whereas *A. rhizogenes* is more closely related to *Rhizobium tropici* than it is to other agrobacteria (109). These conclusions, however, require further support from comparisons of other chromosomal sequences (63).

The *rrnA* operons of two representative *A. vitis* strains (K309, with an O/C, large TA Ti plasmid, and strain S4, with a vitopine Ti plasmid) have been completely sequenced and are the first such sequences for *Agrobacterium* spp. (66, 67). K309 is the *A. vitis*–type strain and is maintained as NCPPB3554. It should be stressed that it is important in *rrn* studies to compare orthologous copies, a requirement seldom taken into account when ribosomal sequences are used to construct phylogenetic trees. Since ribosomal RNAs are considered to be highly conserved within a species, it came as a surprise that the so-called B8 stem-loop structure at the 5′ end of the 23S rRNA of K309 was 142 nt shorter than that of S4. Such features can be used as synapomorphic markers to allow cladistic analysis within and between strains. The 16S–23S intergenic region (IGS) contains highly conserved and more variable regions. Homologies for the sequences of the two *A. vitis* strains vary from 56.7% for the 5′ end of the *rrn* operon to 100% for the three tRNA genes and the 5S gene. About 325 positions within the *rrn* operons of K309 and S4 differ. In the regions of secondary structure, compensatory base-pair changes are found, which confirms the stem-loop models for the RNAs (67). Because of the nonrandom pattern of homology distribution at the *rrn* level, it is important to use the same *rrn* regions (preferably including slowly and rapidly evolving fragments) when results of different studies are compared.

It would be interesting to include the three other *rrn* copies of the *A. vitis* genome in such studies. *rrn* operon evolution involves amplification and possibly

gene conversion (modification of a copy by unilateral sequence transfer from another copy), a phenomenon that must be taken into account when establishing phylogenetic relationships from such sequences. Evidence for gene conversion in *A. vitis* has been obtained in at least two cases: for the AB4 *ipt* gene (65) and for different *tmC* genes (81).

The *A. vitis* ribosomal sequences now available have allowed a detailed comparison of a number of strains by using IGS primers. These studies (61, 68) have shown the potential of this type of analysis to detect small differences between closely related strains, to rapidly classify strains, and to identify new strain types. It was also shown that restriction enzyme analysis of total bacterial DNA (using standard strain patterns as markers) can be used for preliminary identification. Evidence for the existence of diverse chromosomal types in *A. vitis* was obtained from these studies. It was possible to separate tumorigenic *A. vitis* into distinct groups (and sometimes to the strain level) based on DNA fingerprints of an intergenic spacer region (the region between the 16S and 23S rRNA genes) and of the 5′ end of the 23S gene. Although more diversity among strains was detected by RAPD analysis, this method also resulted in strain groupings that were highly correlated with those identified by the rRNA gene fingerprinting. It was determined that well-defined groups exist within the species and that chromosomal type is highly correlated with the type of Ti plasmid carried by strains. For example, 25 of 26 strains (originating from the United States and Hungary) carrying nopaline type Ti plasmids had identical IGS and 5′ 23S fingerprints. Strains carrying vitopine plasmids were divided into three clusters, and those with O/C plasmids formed clusters with large or small TA-regions. Note that PCR-RFLP analysis may not distinguish between point mutations and large-scale events like insertions or deletions of longer DNA stretches; however, these events should be considered separately when a phylogenetic tree is constructed. Further studies are therefore needed to interpret PCR-RFLP patterns in terms of evolutionary change within the *A. vitis* group; these could yield quantitative data on degree of diversity and speed of evolution.

As mentioned previously, only nontumorigenic strains of *A. vitis* were isolated from feral *V. riparia*. When 24 strains were analyzed by IGS fingerprinting, they were highly diverse and generated 13 different fingerprints, none identical to fingerprints of tumorigenic strains (22). It will be interesting to compare in more detail the genomes of tumorigenic and nontumorigenic strains. Such research may lead to a better understanding of factors that influence the apparent Ti plasmid/chromosome specificity and further elucidate the evolutionary development of *A. vitis* as a grape pathogen.

GENES ASSOCIATED WITH HOST-PATHOGEN INTERACTIONS

A. vitis shows a high degree of host specificity for grape. Although other *Agrobacterium* spp. have been identified in grape rhizospheres (17), *A. vitis* is by far the predominant species detected in grape crown galls. Genes that are associated with

host interactions are known to reside on Ti plasmids, on plasmids that encode tartrate utilization, and on the chromosome.

Ti Plasmid Genes

Early molecular studies on *A. vitis* concentrated mainly on the host range properties of two strains of the octopine type (later determined to be O/C types): Ag162 and Ag57 (12, 13, 43, 55, 101, 110). Ag162 and Ag57 differ in host range from the *A. tumefaciens* strain A6 that carries an octopine-type Ti plasmid. Whereas A6 has a wide host range (WHR) that allows tumor induction on common test plants such as *Nicotiana tabacum* or *Datura stramonium*, Ag162 and Ag57 expressed a limited host range (LHR), inducing tumors on *Vitis vinifera* and only a few test plants such as *Lycopersicon esculentum* and *Kalanchoe tubiflora*. Three host range–related genes on the Ti plasmid were identified: *virA*, *virC*, and the T-DNA oncogene *ipt*, which encodes the synthesis of a cytokinin.

Vir *Genes* The virulence system of *A. tumefaciens* has been studied extensively. It comprises a large number of genes located in a compact 30-kb virulence region [for reviews see (50, 88)]. These genes are generally responsible for encoding proteins that liberate the T-DNA from the Ti plasmid and transport it to the plant cells. The *virA* gene encodes a sensory protein that detects the presence of monosaccharides and phenolic compounds such as acetosyringone secreted by wounded plant cells (27). In the presence of these signal molecules, VirA undergoes autophosphorylation and transfers a phosphate group to VirG, which acts as a transcriptional regulator for other *vir* genes (49).

Studies on *virA* of pTiAg162 showed that this gene is partially responsible for the limited host range of *A. vitis* Ag162. Replacement of its *virA* gene with the A6 *virA* enhanced host range (110). The LHR VirA protein has only 45% amino acid homology to the WHR VirA. Further studies demonstrated that domains acting as receptors for the sugar and phenolic signals are conserved in the LHR VirA protein. The LHR *virA* differs from WHR *virA* in that its promoter region is not inducible by acetosyringone (103).

The *virC* gene of WHR strains prevents tumor induction on grapevine by the induction of a hypersensitive response, and *virC*-minus mutants are virulent (110). Although the precise mechanism for the hypersensitive response has not been established, the WHR *virC* gene likely increases the efficiency of T-DNA transfer. On grape this may lead to toxic levels of hormones encoded by the WHR T-DNA oncogenes.

The *virD2* gene encodes an endonuclease that recognizes the T-DNA borders and liberates the T strand, a single-stranded DNA molecule that is subsequently exported to the plant cell. The T strand is protected by the *virE*-encoded single-stranded DNA-binding protein VirE2, which contains a nuclear targeting signal. Transgenic plants producing VirE2 can complement a *virE* mutant, which shows that VirE2 acts in the plant cell probably by protecting and transporting the T

strand. A similar complementation has been achieved for the *virF* gene. This gene is found in octopine strains of *A. tumefaciens* but is lacking in nopaline strains and its precise function is unknown. Recently, *virF* was found in O/C and nopaline strains of *A. vitis* at an unusual position within the *vir* region. The *A. vitis* and *A. tumefaciens virF* genes are highly similar; both contain a *vir* box (conserved region of *vir* genes required for signal-regulated transcription) and are induced by acetosyringone (85).

Finally, the large *virB* operon of *A. tumefaciens* contains 11 open reading frames. A number of these encode proteins that assemble into a sex-pilus and a periplasmic membrane-located transfer complex (50, 88). The *virB* genes of *A. vitis* have not yet been studied.

T-DNA Oncogenes The main tumor-inducing genes of octopine and nopaline strains of *Agrobacterium tumefaciens* are the *ipt* and *iaa* genes. The *ipt* gene encodes an isopentenyl transferase that catalyzes the synthesis of cytokinins. The *iaa* genes encode indoleacetic acid (IAA) synthesis in two steps: *iaaM* encodes the synthesis of indoleacetamide (IAM) from tryptophan, whereas *iaaH* encodes the conversion of IAM into IAA. The LHR strains of *A. vitis* lack the *ipt* genes normally found in *A. tumefaciens* WHR strains, and the introduction of this gene into an LHR strain leads to host range extension (13, 43). It was postulated that cytokinins inhibit tumor development on grapevine (110). The Ti plasmids of both WHR and LHR strains contain *iaa* genes. The *iaa* genes of Ag162 and Ag57 were found to be located on the TB-DNA (12, 110). Later it became clear that other *A. vitis* strains contain an active *ipt* gene and an additional active *iaaM* gene on their TA-region (6, 7) and are of the WHR type (76). Mutation studies of the TA- and TB-regions of the WHR plasmid pTiTm4 (45) showed that the *ipt* gene does indeed inhibit tumor formation when it is not counterbalanced by the combined action of the TB-*iaaM*, TA-*iaaH*, and TB-*iaaH* genes. Although the TB-*iaa* genes are sufficient to induce tumors on grapevine, their capacity to induce IAA synthesis is too weak to overcome cytokinin inhibition by the *ipt* gene (44). The TA-*iaaM* gene provides the necessary amount of IAM, which is converted to IAA by the TB-*iaaH* gene product. In the case of *ipt* gene loss (as has occurred in small TA O/C strains like AB3, Ag162, and Ag57), the TA-*iaaM* gene is no longer needed (and is also found to be deleted in these strains). These studies also revealed the oncogenic action of the TA-*6b* gene that induces tumor formation on grapevine in the absence of *ipt* and *iaa* genes (45). The mechanism of tumor formation by *6b* has not yet been determined, but active *6b* genes are present in all *A. vitis* strains. It was shown that the *6b* genes from different *Agrobacterium* strains have different tumor-inducing capacities and may therefore play a role in host range.

T-DNA Structures

Partial or complete T-DNA sequences have been determined for pTiTm4 (TA and TB), pTiAB3 (TA and TB), pTiAB4, pTiS4, and pTiCG474. Knowledge of these

TABLE 2 References for T-DNA maps of *A. vitis* model strains

Region of T-DNA	Type of Ti plasmid (strains)	Reference
Large TA	O/C (Tm4)	(74)
Large TA	O/C (Hm1)	(78)
Small TA	O/C (AB3, Ag57)	(74)
TB	O/C (Tm4, K305, Ag57, AB3, NW233, Hml)	(37, 71)
Entire T region	N (AB4)	(65)
T1, T2, T3	V (S4)	(26)

sequences has greatly increased our understanding of T-DNA structures in *A. vitis* and facilitates the design of PCR primers for rapid detection and characterization of tumorigenic strains (87). Molecular studies of a large number of *A. vitis* strains has revealed four major types of T-DNA structures (summarized in Figure 1; references for maps in Table 2).

1. The large TA-DNAs of the O/C strains are clearly related to the classical octopine TL-DNA from *A. tumefaciens* strain A6. However, unlike the A6 TL-DNA, they have an intact and functional agrocinopine synthase (*acs*) gene (77), lack the *6a* gene, and carry an IS*866* element within the *iaaH* gene (except strain Hm1) (75). The small TA-regions (73–75) were derived from a large TA-region by an internal deletion that removed the *acs*, *iaaH*, *iaaM*, and *ipt* genes, leaving only the *6b* and *ocs* genes intact. Two additional IS elements, IS*868* and IS*869*, occupy more than one half of the small TA-DNA. The *A. vitis* octopine synthase enzymes also differ in biosynthetic capacities from the A6 enzyme in that they synthesize octopine, lysopine, and octopinic acid but not histopine and methiopine (70).

The Ti plasmids of the *A. vitis* O/C strains harbor a second T-region of about 20 kb, called the TB-region (12, 110). This region carries functional *iaa* genes, an *acs* gene, and the cucumopine synthase (*cus*) gene (also found in some *A. rhizogenes* strains). Cucumopine is a condensation product of histidine and α-ketoglutarate (31). The TB-DNA has not yet been fully sequenced, and it may contain other oncogenes or opine genes. At least six TB-DNA variants have been defined that have insertion elements at different positions (71). The TB-region of Tm4 is shown in Figure 1.

In general, the structures of the *A. vitis* TA and TB-regions have diverged considerably by acquisition of insertion elements like IS*866*, IS*867*, IS*868*, IS*869*, and IS*870* that are also found at other locations in the genome (both in the Ti plasmids and in the chromosomes). They may be associated with *A. vitis* or in a more general way with the Rhizobiaceae. Numbers and positions of these elements can be used to reconstruct the evolution of both plasmids and chromosomes (78). Evidence for clonal strain origin is provided by a detailed study of the TA-DNA

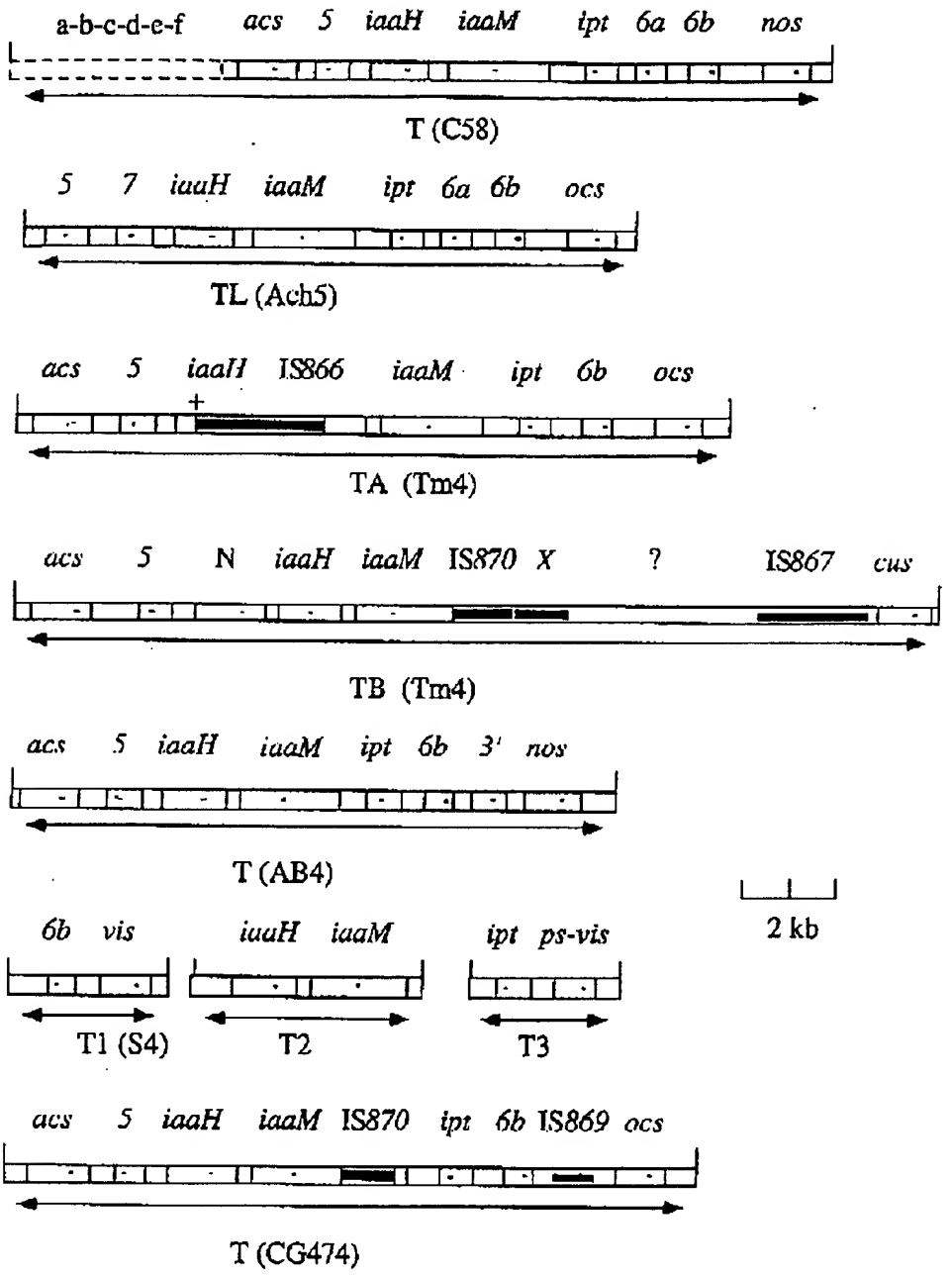

Figure 1 Structural differences of *A. tumefaciens* and *A. vitis* T-DNAs. From top to bottom: TA-DNA from nopaline (C58) and octopine (Ach5) *A. tumefaciens* strains. T-DNAs from *A. vitis* strains include TA-DNA from the octopine/cucumopine strain Tm4; TB-DNA from Tm4; T-DNA from the nopaline strain AB4; T1, T2, and T3 from vitopine strain S4; T-DNA from the octopine strain CG474. Sequenced regions are indicated by *double-headed arrows*.

of the O/C strain Hm1. This TA-DNA is very similar to the O/C large TA-DNAs but carries an intact, biologically active TA-*iaaH* gene (76). This suggests that the other WHR large TA O/C strains, all of which contain an IS*866* element inserted at the same position within the TA-*iaaH* gene, are derived from a single mutant that arose recently. Since O/C strains with large TA-regions have been isolated from vineyards in widely different parts of the world, it appears that this mutant has been effectively disseminated (possibly through trade of contaminated plant material). Note that the TA-*iaaH* gene interruption might be selectively neutral and linked to an unidentified selective character. Recent clonal origins have also been proposed for subtypes of the O/C Ti plasmids (37), the *A. vitis* nopaline (65), and vitopine (39) Ti plasmids.

2. The nopaline T-DNA of AB4 is found without variation in most *A. vitis* nopaline strains and is similar to the right-hand part of the classical nopaline T-DNA of *A. tumefaciens* C58. It lacks the 11.5-kb left-hand fragment of the pTiC58 T-DNA containing genes *a–f*. The functions of genes *a–f* are unknown, but gene *e* plays an important role in tumor induction by C58 (11). Apparently, AB4 can compensate for the loss of gene *e*.

Genes *6a* and *6b* found on the C58 T-DNA are replaced in AB4 by a fragment (F) carrying a distantly related *6b* gene and a gene called *3'* (the latter was first identified on the TR-DNA of *A. tumefaciens* octopine strains such as A6). Both genes have oncogenic properties (L Otten, unpublished data). Since a complete, C58-like nopaline T-DNA with fragment F has been found in pTi82139 (carried in *A. rhizogenes* strain 82139) (33), and since the *vir* region of AB4 to the left of the T-DNA is very similar to the O/C *vir* region, the *A. vitis* nopaline T-DNA probably results from a fusion between a pTi82139-like T-DNA and an O/C large TA-DNA. The precise fusion point has not yet been established.

3. The pTiS4 plasmid is unusual in that it contains three T-DNAs, the T1-, T2-, and T3-DNA, each with a single type of oncogenic function (*6b*, *iaaH*/*iaaM*, and *ipt*, respectively (26). The homology of these genes with their counterparts in other Ti plasmid types is surprisingly low, ranging from 58 to 62% of sequence identity. In contrast, homologies between genes from other T-DNAs is about 90–95%. This suggests that the T-DNA oncogenes have evolved over a longer period than was originally suspected. The T1-DNA carries the vitopine synthase (*vis*) gene, which is related to the octopine synthase gene. The structure of vitopine has not yet been determined.

4. Finally, the T-DNA of the unique isolate CG474 resembles the classical octopine TL-DNA and O/C TA-DNA (L Otten, unpublished data) but has a number of characteristic differences. It carries an IS*870* element in the *iaaM* gene (thus inactivating *iaa* synthesis) and an IS*869* element between the *6b* and *ocs* genes. The *acs* gene is intact and *6a* is missing. It is remarkable that this strain induces tumors on grapevine, in spite of an active *ipt* gene and the lack of an *iaa* system. Possibly, the *ipt* gene is less active than in the large TA O/C strains or is rendered less toxic by the *6b* gene. Several *6b* genes facilitate tumor formation in the presence of an inhibitory *ipt* gene (102).

Tartrate Utilization Plasmids

Most *A. vitis* strains degrade tartrate, an abundant compound in grapevine. Tartrate utilization can easily be tested by growth on minimal medium with 0.5% tartrate. It has been shown by conjugational plasmid transfer and DNA transformation that utilization of tartrate is plasmid-encoded in most of the strains (99). Three tartrate plasmid types, all of which are conjugative in planta, have been defined; pTrAB3 (245 kb), pTiAB3 (234 kb), and pTrAB4 (170 kb). pTrAB3 integrates readily into the chromosome of an acceptor strain together with another 60-kb plasmid from AB3 (64). The importance of this phenomenon under natural conditions is unknown but may lead to stabilization of the plasmid. The restriction maps of the tartrate utilization plasmids have been determined and the essential tartrate degradation regions (called TAR-I for pTrAB3, TAR-II for pTiAB3, and TAR-III for pTrAB4) have been analyzed by transposon mutagenesis using the *uidA*-Tn*5* transposon, which allows detection of transcriptional units. The TAR regions are similar in sequence (81); they are 11 kb in size and carry, among other genes, two tartrate dehydrogenase (*ttuC*) genes that were identified on the basis of homology to a *Pseudomonas ttuC* homologue. Like Ti plasmids, TAR regions are generally associated with characteristic chromosomal backgrounds (81). A few *Agrobacterium* grapevine strains use tartrate but lack classical TAR sequences, which indicates the existence of other tartrate utilization systems (81). Figure 2 presents the map and the functional organization of the TAR-I region of pTrAB3.

Remarkably, the three TAR regions found on otherwise unrelated plasmids are possibly the result of horizontal DNA transfer. The ends of the TAR regions show no evidence for insertion sequences so that the *ttu* genes do not seem to be part

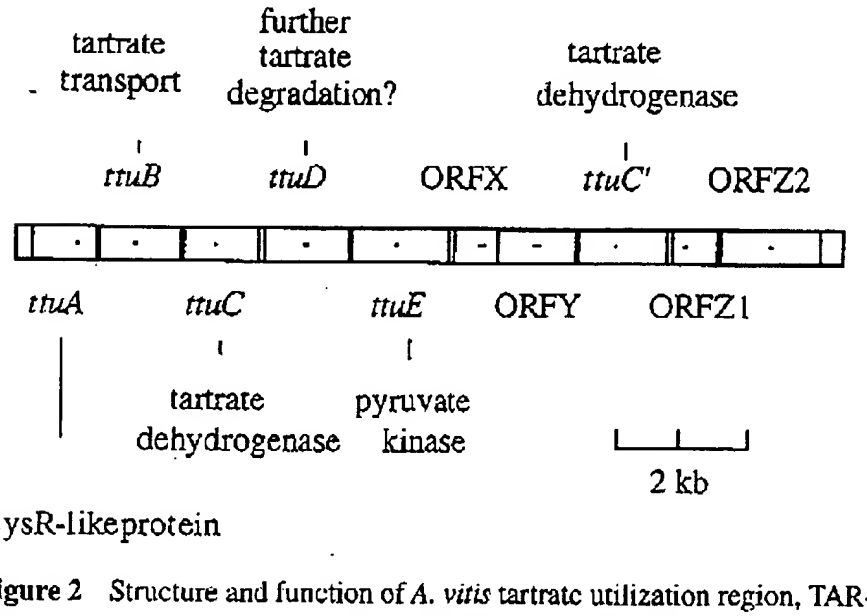

Figure 2 Structure and function of *A. vitis* tartrate utilization region, TAR-I (81, 82). This region is highly similar to that of TAR-II and TAR-III.

of a transposon. O/C strains with a small TA-DNA, like AB3, carry a pTiAB3-like plasmid with the TAR-I region and a Ti plasmid with a second TAR region (TAR-II). The fact that the pTiAB3-like plasmids carry a TAR region strongly suggests that exploitation of grapevine tissues through tartrate utilization and tumor formation is part of the same strategy and that such a Ti plasmid may constitute an efficient "grapevine colonization package." Indeed, a few *A. tumefaciens* strains isolated from grape carry a pTiAB3-like plasmid, the only type to contain both functions. Furthermore, competition experiments done in planta with one such strain and its tartrate-minus derivative showed that the TAR region provides a selective advantage for grape colonization (82).

Present evidence therefore suggests that tartrate utilization provides a competitive advantage to *A. vitis*. However, it was recently determined that most (23 of 26 strains) of nontumorigenic *A. vitis* isolated from feral grapevines do not utilize tartrate, which suggests that these strains possess other host colonization factors (22). It would be interesting to know whether other grape-associated bacteria, like *Xanthomonas ampelina*, carry tartrate plasmids and similar tartrate utilization genes.

Chromosomal Genes

Other potential host specificity–related factors include the production of polygalacturonase (PG) and endoglucanase (60). PG, which has been studied more extensively, is associated with induction of grape necrosis and was identified as a virulence factor since a PG-minus mutant was impaired in ability to induce tumors on grape and to attach to grape roots (10, 80). The *pehA* gene from *A. vitis*, which encodes for PG, was cloned and sequenced and the enzyme was compared to PGs from other microorganisms. The *A. vitis* enzyme is more similar to PGs produced by the plant pathogenic bacteria *Ralstonia solanacearum* and *Erwinia carotovora* than it is to those from *Aspergillus niger* and *Lycopersicum esculentum* (42) The *A. vitis* PG released dimers, trimers, and monomers from polygalacturonic acid and caused less electrolyte leakage from potato tubers than did PGs from *E. carotovora* and *R. solanacearum*.

Another chromosomal gene of *A. vitis* that may be associated with host interactions is homologous to *rfbC*, which encodes dTDP-rhamnose dehydratase that may be associated with EPS capsule biosynthesis (TC Herlache & TJ Burr, unpublished data). Primers derived from this gene amplify a 167-bp product from all *A. vitis* strains but not from *A. tumefaciens* or *A. rhizogenes* (61). Although its role in host interactions has not been identified, it is speculated that this gene may be associated with attachment of *A. vitis* to grape.

Although much progress has been made toward identifying genes that are associated with *A. vitis*-grape interactions, further research is needed to determine more specifically their roles under natural conditions. For example, the genetic makeup of LHR and WHR *A. vitis* T-DNAs is known and phenotypes can be differentiated on indicator plants. However, how do differences in T-DNAs affect grape

host range or other interactions under natural conditions where pathogenicity of both groups appears to be limited to grape? Another example is gene *pehA*, which encodes a PG that is hypothesized to facilitate *A. vitis* attachment and systemic colonization of grape. However, nontumorigenic *A. tumefaciens* strains do not carry the *pehA* gene and are also common grape endophytes (TJ Burr, unpublished data).

Other observations also illustrate the difficulty of utilizing controlled inoculation experiments to study how Agrobacterium interacts with host plants in nature. For example, although tumorigenic *A. tumefaciens* are rarely isolated from grape crown galls, the bacterium causes large galls on grape shoot explants in vitro, even on cultivars known to be crown gall resistant (TJ Burr, unpublished data). In contrast, when grape shoot explants are inoculated with *A. vitis*, necrosis develops in 24–48 h (discussed below). Therefore, identifying genes and their functions that confer natural host specificity will be a great challenge but should provide key information to develop novel approaches for disease control.

A. VITIS-INDUCED GRAPE NECROSIS

A. vitis typically causes gall formation on grape trunks at or above graft unions. It is most interesting that galls are rarely observed on roots, but instead the bacterium induces a grape-specific necrosis. Tumorigenic and nontumorigenic *A. vitis* strains induce necrosis within 24–48 h on roots of all *Vitis* species, but not on other plants that have been examined (15, 95). Recently, it has been determined that necrosis is also induced on shoot explants and grape leaves, that induction is inoculum dependent, and that genes for necrosis are carried on the bacterial chromosome (TJ Burr, unpublished data). Therefore, *A. vitis*–induced necrosis is different from the necrosis reported for certain *A. tumefaciens* strains, which appears to be related to hormone toxicity and is associated with different Ti plasmid genes (79, 110).

As mentioned above, PG has been identified as one necrosis factor. A PG-minus derivative of strain CG49 (CG50) was shown to induce less necrosis at concentrations of 10^6 cfu/ml. Since higher concentrations of CG50 induced necrosis, it was concluded that factors other than PG were involved. Recently, it was shown that a *pehA*-complemented CG50 produced more necrosis than the noncomplemented mutant, but significantly less than CG49 (TC Herlache & TJ Burr, unpublished data). These results have encouraged further research on the necrosis mechanism.

It was recently discovered that *A. vitis* induces a hypersensitive response (HR) on tobacco and that both HR and grape necrosis can be blocked by preinoculation with certain plant metabolic inhibitors and may be mechanistically related (TC Herlache & TJ Burr, unpublished data). Necrosis and HR reactions therefore require active plant metabolism. Like HR reactions of other gram-negative bacteria, the *A. vitis* HR has an induction period in tobacco (8–12 h) prior to which the reaction can be stopped by infiltrating leaf panels with antibiotics. HR- and necrosis-associated genes are being cloned and sequenced.

SURVIVAL IN SOIL AND PLANT TISSUE

Although bacteria in the genus *Agrobacterium* are common soil inhabitants, tumorigenic strains are almost exclusively detected in association with galls and plant residues in soil (3, 17). Following infestation of soil with *A. vitis*–colonized grape roots and canes, the bacterium was detected in the tissues for at least two years (25). The roots and canes did not have galls, however, although all of the recovered strains were still tumorigenic and carried O/C, N, or V-type Ti plasmids. Therefore the bacteria are able to maintain their Ti plasmids while persisting in an apparent saprophytic state in decaying grape tissues. In another study where grapes or oats were planted in soils infested with *A. vitis*, the bacterium maintained greater populations in grape as compared with oat rhizospheres (5). These and other studies infer that *A. vitis* may not persist in soils in the absence of grape tissues and that the bacterium is primarily introduced into soils with contaminated grape plants. However, because the sensitivities of methods for detecting the bacterium in soil are low (generally populations $<10^2$–10^3 cfu/g soil cannot be detected) and because of the recent findings that are discussed below, further investigations of soil survival are necessary.

As mentioned previously, studies were undertaken in the United States (22) and Italy (C Bazzi, unpublished data) to determine if *A. vitis* persists in feral *Vitis* spp. *A. vitis* was detected from 41 of 66 root samples collected from regions near to and far removed from commercial vineyards in New York and Vermont. All strains that were identified as *A. vitis* were nontumorigenic and were genetically diverse, as determined by DNA fingerprinting. In Italy, over 50 strains of nontumorigenic *A. vitis* were recovered from cuttings taken from feral *V. vinifera silvestris* vines. Since the feral vines probably grew from seeds that were disseminated by animals, one must question the source of *A. vitis* on them. It may be that *A. vitis* is a relatively common inhabitant of soils, but can only be detected by conventional plating methods at populations achieved in association with grapevines. Further research is therefore needed to determine if *A. vitis* survives in soil at low population densities. The ability to make this determination will be, at least partially, dependent on the development of detection methods having increased sensitivities.

Important questions remain on the role of feral grapevines in the survival of *A. vitis* and the spread of crown gall disease. Do tumorigenic strains colonize feral grapevines, and can the nontumorigenic strains that are present acquire and maintain Ti plasmids? To answer the second question, we mated tumorigenic strains (carrying O/C and N Ti plasmids) with two nontumorigenic strains from feral *V. riparia* and with one from *V. vinifera* (TJ Burr, unpublished data). Matings were done on grape galls that were caused by the donor strain (to assure the presence of proper opines). Equal concentrations of donor and recipient (rifampicin resistant) were incubated on galls, and selection for Ti plasmid transfer was done on media containing rifampicin and the appropriate opine as the sole N and C source. From six matings (338 colonies selected from opine media), no stable Ti plasmid transfer to strains from *V. riparia* was detected, and the O/C Ti plasmid

was transferred to a strain of *V. vinifera* in one mating. In two cases, evidence of a transferred Ti plasmid was initially detected by Southern analysis, however after further evaluation the plasmid could no longer be detected. Additional research is needed to identify factors that are associated with the apparent inhibition of Ti plasmid transfer. Interestingly, transconjugants were frequently identified that had gained a plasmid (other than Ti) that conferred tartrate utilization from donor strains.

Endophytic Survival

Lehoczky first demonstrated that *A. vitis* survives endophytically in apparently healthy grapevines and initiates gall formation at injury sites (57). In a series of papers, he demonstrated the presence of the bacterium in grape sap (58) and hypothesized that bacterial cells concentrate in the grape root system in winter and move into the upper parts of the vine during sap flow (59). In fact, *A. vitis* is routinely isolated from root tissues, even during the growing season; however, the frequency of isolation from roots has not been compared with that from other tissues. Although it has been generally assumed that *A. vitis* persists primarily in xylem tissues, at least two studies indicate that the bacterium may reside in tissues other than the xylem. Süle reported that the highest *A. vitis* populations were detectable in phloem tissue (93), and Jager et al (47) provided evidence that the bacterium persists in the rind layer of dormant cuttings (directly below the bark).

Bauer et al examined the seasonal distribution of *A. vitis* in vines in Germany (1). In this case, *A. vitis* was detected primarily at the inoculation sites on canes and did not readily disseminate throughout the vine. By using antibiotic-marked strains it was, however, possible to detect *A. vitis* cells that migrated from the inoculation points to roots within 15 weeks. Populations at inoculation sites showed seasonal fluctuations (they were highest in late May and in October) and differed between cultivars; they were highest in Riesling as compared with Müller-Thurgau. Stover et al also found that *A. vitis* does not readily migrate from inoculation points and that populations differed between resistant and susceptible cultivars (91). He also showed that freezing of canes facilitates the systemic movement of *A. vitis* (a 41,000-fold increase in cells was detected after flushing water through freeze-treated canes). Since there were no differences in total numbers of cells detected in frozen vs nonfrozen canes, it was concluded that freezing played a physical role in allowing the bacteria to move within the cuttings. If one considers that *A. vitis* may reside in phloem or rind tissues, then freeze injuries may facilitate its spread into xylem tissues and enhance dissemination within the vine. The finding that freezing facilitates systemic movement has potential implications for improving the sensitivity of indexing methods (discussed below). It also suggests that freezing injuries may be important not only for stimulating the secretion of *Agrobacterium* virulence gene inducers from injured cells (discussed below), but may also facilitate the internal movement of the bacterium in vines.

MANAGEMENT OF GRAPE CROWN GALL

Grape growers have dealt with crown gall disease for over 100 years and recognized early on that cultivars differ in susceptibility to disease and to factors that cause vine injury, particularly freezing temperatures, which stimulates disease development (41). Some cultural practices that can affect vine injury and thus crown gall development include vineyard site selection, crop and canopy management, and the protection of trunks with soil during winter months. When practical, the selection of resistant rootstock and scion cultivars can also greatly influence disease development. Vitis germplasms have been evaluated for crown gall susceptibility by different research groups (36, 90); in general, rootstocks of *V. riparia* and *V. rupestris* parentage are more resistant than *V. vinifera* cultivars. The genetic basis for resistance was investigated by screening progeny from crosses between resistant (*V. amurensis*) and susceptible cultivars. Following inoculation with *A. vitis* strain AT-1, it was concluded that resistance is controlled by a single dominant gene (97). It remains to be determined if the same pattern of resistance will be observed on other grape progeny when inoculated with a genetically diverse collection of *A. vitis* strains.

Süle et al (94) compared crown gall severity under field conditions on a highly susceptible scion cultivar, Blue Frank, that was grafted onto either crown gall–resistant (Riparia 'Gloire') or –susceptible (Teleki 5C) rootstocks. Over a six-year period, significantly more crown gall and vine death occurred on vines that were grafted on the susceptible rootstock. This report is particularly significant in that it is the first under controlled conditions in the field to demonstrate a beneficial effect of using crown gall–resistant rootstocks. The reduction in disease may be related to reduced survival of *A. vitis* in resistant as compared with susceptible grape genotypes (91). Other research on crown gall management has focused on developing methods to index vines, producing propagation material that is free of the pathogen, and on biological control. A review of disease management strategies was recently published (14).

Indexing for *A. vitis*

Lehoczky demonstrated the presence of *A. vitis* in symptomless dormant cuttings by isolating the bacterium from callus tissue that formed at the base of cuttings (58). Variations of this method have been used successfully by others to index cuttings (93), although its sensitivity has not been determined. Another indexing method, developed by Tarbah & Goodman (100) and by Bazzi et al (2), involves forcing water or buffer through cuttings with vacuum pressure and isolating the pathogen from the collected extracts. *A. vitis* is then identified by plating the extracts on semiselective media (18) and/or by serology (4). The efficiency of the method was evaluated by infiltrating cuttings with known concentrations of *A. vitis* and then calculating the percentage of recovered cells (2). Because only about 12% of the introduced cells were detected, the method would appear to have limited value for

general indexing of propagation wood sources. As mentioned above, freezing of cuttings prior to flushing with water greatly increases the recovery of *A. vitis* and therefore may significantly improve the efficiency of this method.

Increased emphasis has been placed on the use of DNA probes and PCR (32) to identify *Agrobacterium* spp. Probes have consisted of chromosomal and T-DNA genes and IS elements (20, 63, 87). Sawada et al (83) compared primers derived from conserved regions of *virG*, *virC1*, and *virC2*. A primer pair including a forward primer (VCF) from *virC1* and a reverse primer (VCR) from *virC2* consistently gave a 730-bp PCR product from 75 of 77 strains of tumorigenic species including *A. tumefaciens*, *A. rhizogenes*, *A. rubi*, and *A. vitis*. When other primers consisting of forward primers from *virG* and reverse from *virC* genes were used, some strains did not yield products and others yielded products of unexpected sizes.

Similarly, Haas et al developed primers derived from the endonuclease portion of the *virD2* gene and from a conserved region of the *ipt* gene (40). In this case, 44 strains representing *A. tumefaciens*, *A. rhizogenes*, and *A. vitis* all yielded the expected 338-bp product from the *virD2* primer, whereas nontumorigenic strains did not. The *ipt* primer pair also produced an expected product size from tumorigenic strains but, as expected, not from rhizogenic strains or from a LHR *A. vitis* strain. Using pure cultures of bacterial cells, this PCR procedure could detect reaction mixtures containing as few as 150–200 cells.

There is continued development of PCR-based methods for *A. vitis* and other *Agrobacterium* spp. that utilize specific primers and novel methods for isolating bacteria and bacterial DNA from plants and soil. Eastwell et al (35) tested different methods of extracting bacterial DNA from dormant cuttings and compared primers for their effectiveness in detecting *A. vitis*. Primers were derived from the *pehA* gene (polygalacturonase gene) and from *virA* of the Ti plasmid. The method was effective in detecting *A. vitis* in grape cuttings collected from grapevines expressing crown gall. In this case, *pehA* primers amplify a characteristic product from all *A. vitis* (tumorigenic and nontumorigenic), and *virA* primers amplify a characteristic product from *A. vitis* strains carrying N and O/C but not V Ti plasmids. To overcome this problem, we recently developed a *virE2* primer that amplifies a characteristic product from strains with V Ti plasmids (61). The ability to detect vitopine strains in cuttings is essential because they make up a significant portion of *A. vitis* strains found in grape (68). The *pehA* primers are highly reliable for identifying *A. vitis*, although recently a naturally occurring PG-negative strain of *A. vitis* was isolated from *V. riparia* (TJ Burr, unpublished data). However, where a grape grower wishes to know if propagation material is contaminated with tumorigenic *A. vitis*, exclusive reliance on *pehA* primers will not be sufficient. Nontumorigenic strains, which carry *pehA*, are often encountered in *Vitis* spp., and at least some nontumorigenic strains may actually benefit the plant by preventing tumorigenic strains from causing infections (discussed below). In place of *pehA* primers, we recently evaluated primers derived from the *rfbC* homologue that was previously discussed (61).

Another recently developed method to detect *A. vitis* in grapevines involves the use of an immunocapture technique, followed by PCR (51). In this case, extracts from grape canes are incubated in antibody-coated tubes to which 3DG medium (selective for *A. vitis*) is subsequently added. DNA from the resulting bacteria is analyzed by PCR with primers derived from the *6b* gene of the *A. vitis* O/C strain Tm4. By this method, a characteristic PCR product was observed for samples known to be infected with *A. vitis* carrying O/C-type Ti plasmids. The authors suggested improvements by employing antibodies and PCR primers that would detect diverse types of *A. vitis*.

The major components of a method for efficient and sensitive detection of tumorigenic *A. vitis* in dormant cuttings seem to be in place. Further studies are needed to compare some of the most promising techniques described above and to compare PCR primers to assure that they will amplify characteristic products from the diverse genetic groups of *A. vitis*. It may be that *A. vitis*-specific primers, such as those from *pehA*, or derived from the *rfbC* gene together with universal primers for tumorigenicity, (such as those reported by Haas et al or Sawada et al) would be highly effective.

A. vitis–Free Propagation Material

The submersion of dormant grape cuttings in a 50°C water bath for 30 min to eradicate *A. vitis* was first reported in 1989 (21). Although initial findings indicated that treatments were effective, a more critical set of experiments demonstrated that low levels of the bacterium survived in tissues near galls even after treatments of 55°C for 30 min (23).

A significant drawback to the use of hot water treatments is the potential for bud injury. Factors including temperature and duration of treatment, the month when cuttings were collected (differences in state of dormancy), and pre- and posttreatment storage were evaluated on cultivar Cabernet Sauvignon by Wample in Washington State (105, 106). He demonstrated that cuttings collected in January and stored posttreatment had the greatest bud survival following hot water treatments. Good bud survival was achieved even following a treatment of 56°C. The use of such temperatures, which are greater than previously tested, to eradicate *A. vitis* may improve the efficacy, although this has not been evaluated. In contrast, using temperatures above 50°C on cuttings collected from vineyards in other areas, such as New York State, has led to significant bud kill (TJ Burr, unpublished data). The reasons for these apparent differences in bud sensitivity to treatments conducted in different geographic regions are unknown but may be related to cultivar differences or to wood hardiness. Therefore, the usefulness of hot water treatments as a crown gall management strategy needs further evaluation. Recent findings suggest that hot water treatments may be warranted even if they do not completely eliminate *A. vitis*. For example, it was observed in Italy and in a commercial nursery in Washington State that hot water–treated cuttings have enhanced callus and root formation, which promotes better plant growth (C Bazzi

& TJ Burr, unpublished data). Such benefits have resulted in the routine treatment of several million cuttings annually in some nurseries.

The only proven way of producing *A. vitis*-free grapevines is by propagating vines from shoot tips in vitro. *A. vitis* does not systemically invade green grape shoots (19, 98), and therefore it is possible to exclude the bacterium by shoot tip culture. This practice has been used by at least one commercial vineyard to establish a crown gall–free mother block that has remained free of crown gall for over five years. Establishment of such a planting requires the selection of a site where the soil is not contaminated with *A. vitis*. Problems of assuring that soils are free of the bacterium were discussed previously.

Biological Control

Several laboratories have attempted to identify biological control agents for grape crown gall (54; reviewed in 14). One promising strain, F2/5, is a nontumorigenic *A. vitis* that was isolated in South Africa (89). F2/5 produces an antibiotic that inhibits growth of many tumorigenic *A. vitis* strains in vitro; however, antibiotic-minus mutants of F2/5 were found to be as effective as the wild-type strain for controlling crown gall (24). We are continuing to study the mechanism(s) by which F2/5 inhibits tumorigenesis on grape. Some nontumorigenic *A. vitis* strains from feral *V. riparia* were as effective as F2/5 for inhibiting crown gall caused by strain K306 (22). Subsequently, one strain, CG523, was compared with F2/5 for controlling several tumorigenic strains representing the major *A. vitis* groups. Biological control agents were applied to inoculation sites either 24 h before or simultaneously with the pathogen. F2/5 was more effective for inhibiting gall formation than was CG523. Although some of the tumorigenic strains were not inhibited by F2/5 following simultaneous inoculations, control was enhanced if F2/5 was applied 24 h prior to the pathogen.

Genetic Engineering for Crown Gall Resistance

In recent years, the ability to genetically transform *Vitis* spp. has greatly improved (56), and the prospects for using genetic engineering to confer resistance to bacterial pathogens in plants look promising (34). Because crown gall is an important disease with few disease control options, the use of transgenic approaches for control is an attractive alternative. A main goal will be to engineer resistance into long-standing, high-quality wine cultivars that are crown gall susceptible and are not likely to be replaced by conventional breeding. One strategy currently being tested involves expressing a deletion mutant of the Ti plasmid *virE2* gene in plants. It was previously determined that expression of some *virE2* mutant genes in tobacco results in plants that are resistant to infection by *A. tumefaciens*, possibly as a result of inhibition of T-strand nuclear import (30). Following the expression of a *virE2* mutant in grape, transgenic lines with significant resistance to crown gall were identified (TJ Burr, unpublished data). Other transgenic strategies for crown gall resistance are sure to follow.

CONCLUSIONS

Although crown gall continues to be a serious problem on grape, significant progress has been made toward understanding pathogen biology and disease control. Characterization of worldwide collections of *A. vitis* has provided significant insight into genetic diversity within the species and its relatedness to other Rhizobiacea. Substantial Ti plasmid sequence information has further enhanced our understanding of diversity within the bacterium and has allowed the prediction of phylogenetic relationships. Sequencing has also facilitated the identification of specific genes that are essential for tumorigenicity and other bacterium-host interactions. Sequence information, together with PCR-RFLP protocols, make it possible to develop specific detection methods, which, in addition to having commercial value, will be essential to answer important epidemiological questions related to pathogen survival and spread in nature. For example, does *A. vitis* survive in soil? How do nontumorigenic strains differ from tumorigenic strains? Can nontumorigenic strains from feral grapevines acquire Ti plasmids? If not, what factors affect plasmid transfer?

In comparison with the genetic makeup of Ti plasmids, much less is known about specific chromosomal genes and their functions. DNA fingerprint comparisons have revealed that *A. vitis* chromosomes are diverse and are highly correlated with the Ti plasmid type carried in strains. Further research is needed to determine the roles of chromosomal genes that are associated with phenotypes such as grape necrosis, tobacco HR, and the ability of some nontumorigenic strains to inhibit crown gall infections.

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Expression of a crown gall biological control phenotype in an avirulent strain of *Agrobacterium vitis* by addition of the trifolitoxin production and resistance genes

Thomas C Herlache and Eric W Triplett*

Address: Department of Agronomy, University of Wisconsin-Madison, 1575 Linden Drive, Madison, WI 53706 USA

E-mail: Thomas C Herlache - tcherlache@facstaff.wisc.edu; Eric W Triplett* - triplett@facstaff.wisc.edu

*Corresponding author

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Abstract

Background: *Agrobacterium vitis* is a causal agent of crown-gall disease. Trifolitoxin (TFX) is a peptide antibiotic active only against members of a specific group of α-proteobacteria that includes *Agrobacterium* and its close relatives. The ability of TFX production by an avirulent strain of *Agrobacterium* to reduce crown gall disease is examined here.

Results: TFX was shown to be inhibitory *in vitro* against several *A. vitis* strains. TFX production, expressed from the stable plasmid pT2TFXK, conferred biological control activity to an avirulent strain of *A. vitis* F2/5, against three virulent, TFX-sensitive strains of *A. vitis* tested on *Nicotiana glauca*. F2/5(pT2TFXK) is significantly reduces number and size of galls when co-inoculated with tumorigenic strain CG78 at a 10:1 ratio, but is ineffective at 1:1 or 1:10 ratios. F2/5(pT2TFXK) is effective when co-inoculated with tumorigenic strain CG435 at 10:1 and 1:1 ratios, but not at a 1:10 ratio. When F2/5(pT2TFXK) is co-inoculated with CG49 at a 10:1 ratio, the incidence of gall formation does not decline but gall size decreases by more than 70%. A 24 h pre-inoculation with F2/5(pT2TFXK) does not improve biological control at the 1:10 ratio.

Conclusions: TFX production by an avirulent strain of *Agrobacterium* does confer in that strain the ability to control crown gall disease on *Nicotiana glauca*. This is the first demonstration that the production of a ribosomally synthesized, post-translationally modified peptide antibiotic can confer reduction in plant disease incidence from a bacterial pathogen.

Background

Agrobacterium vitis strains are causative agents of crown gall, an economically important disease [1,2]. *A. vitis* F2/5 is an effective biological control agent against many *A. vitis* tumorigenic strains [3]. Strain F2/5 produces an antibiotic toxic to many *A. vitis* strains *in vitro*. However, two lines of evidence suggest that this antibiotic plays a minor role in disease suppression. Strains that are susceptible to the antibiotic *in vitro*, such as *A. vitis* strain CG78, are able to infect the plant in the presence of F2/5 [3], and Tn5 mutants of F2/5 lacking F2/5 antibiotic production appear to be unaffected in crown gall biological control [4]. Biological control by F2/5 is grape-specific, as F2/5 is not effective on non-grapevine host plants such as *Nicotiana glauca*. Furthermore, F2/5 is not effective against all *A. vitis* strains [3]. Thus, enhancing the F2/5 biological control phenotype and extending the host range of the efficacy of F2/5

beyond grape would be beneficial for disease control in field applications.

Agrobacterium rhizogenes strain K84 is the most studied crown gall biological control strain and is commercially utilized for crown gall disease control worldwide. Strain K84 biological control is primarily due to production of two plasmid-encoded antibiotics, agrocins 84 and 484, encoded by genes on pAgK84 and pAgK434 respectively [5]. Agrocin 84, an adenosine analog [6], is effective against tumorigenic strains carrying nopaline/agrocinopine tumor-inducing plasmids, and requires the *acc* system in the target strain for activity [7]. Agrocin 434, a di-substituted cytidine analog, is effective against, and specific for, a broad range of *A. rhizogenes* strains [8]. Curing of either agrocin-encoding plasmid results in reduction of biological control activity [9]. Thus, K84 demonstrates the efficacy of antibiosis for crown gall biological control. However, the commercial application of the K84 biological control system is limited where *Agrobacterium* strains of certain crops are not inhibited by K84. As a result, alternative biological control systems for crown gall disease are needed.

Trifolitoxin (TFX) is a peptide antibiotic produced by *Rhizobium leguminosarum* bv. trifolii T24. Antibiotic production and resistance functions are encoded by the seven-gene *tfx* operon and the unlinked *tfuA* gene [10,11]. TFX is derived from post-translational cleavage and modification of the *tfxA* gene product. TFX effectively inhibits growth of members of the α-proteobacteria including strains of *Brucella, Ochrobactrum, Rhodobacter, Rhodospeudomonas, Rhizobium,* and the etiological agent of crown gall disease, *Agrobacterium*[12]. TFX is highly specific for this group, as demonstrated by a lack of observable effect on the majority of the bacterial population in the bean rhizosphere [13]. This suggests that TFX could be used to control crown gall diseases of various plants with limited effects on non-target bacteria outside of that very specific group of TFX-sensitive α-proteobacteria. TFX is rapidly degraded in nonsterile soil but is readily extractable from sterile soil inoculated with a TFX-producing strain, suggesting that TFX is sensitive to *in situ* proteolysis [14]. Despite this rapid turnover, TFX production confers enhanced nodulation efficiency upon TFX-producing *Rhizobium* strains under field conditions [15].

Degradation of antimicrobial peptides such as cecropin B and attacin E also has been observed in plant apoplastic fluids [5,16,17]. This is likely due to apoplastic proteinases [5,16]. Expression of antimicrobial peptides in plants has had mixed results for enhancing disease resistance. For example, cecropin expression in transgenic tobacco did not confer resistance to *P. syringae* pv *tabaci*[18], likely due to low apoplastic peptide concentrations due to proteolysis [19]. Therefore, prior to this work it was not clear whether a peptide antibiotic could play a role in the inhibition of galling by tumorigenic *Agrobacterium in planta.*

Agrobacterium rhizogenes is capable of producing TFX by addition of the stable plasmid, pT2TFXK, which contains the *tfx* operon but not *tfuA*[11,12,20]. This suggests that TFX production by crown gall biological control strains of *Agrobacterium*, such as *A. rhizogenes* K84 and *A. vitis* F2/5, may be enhanced by TFX production. These strains would be excellent delivery vehicles for TFX to the infection court.

Here we present experiments demonstrating the TFX-sensitivity of a range of *A. vitis* strains and the effect of the TFX production and resistance phenotypes on the expression of biological control of crown gall using the model plant *Nicotiana glauca.* Strain F2/5 was chosen as the recipient of the TFX genes in this work because it is an avirulent strain that expresses a biological control phenotype on only one host. As a result, F2/5 is an ideal strain in which to test the ability of the TFX system to confer biological control of crown gall disease and to broaden the host-range efficacy of a known biological control strain.

Results

In vitro TFX antibiosis against A. vitis

Agrobacterium vitis strains (Table 1) were tested for sensitivity to trifolitoxin. As expected based on previous results [12], the tested *Agrobacterium vitis* strains were sensitive to TFX-producing strains. However, the level of susceptibility was lower than predicted based upon previous TFX sensitivity measurements with CG48 and CG74 [12]. No zones of *A. vitis* growth inhibition were observed around *R. leguminosarum* T24 colonies, and only relatively small zones were observed around *R. etli* CE3(pT2TFXK), which produces more TFX than T24. Furthermore, one *A. vitis* strain, F2/5, was TFX-resistant.

Because *A. vitis* F2/5 produces an antibiotic to which most of the tumorigenic strains are sensitive [3], the effect of TFX on *A. vitis* was assessed against TFX-producing and non-producing *Rhizobium* strains. None of the tested strains were sensitive to *R. leguminosarum* T24, which produces relatively low amounts of TFX. All of the *A. vitis* strains except for F2/5 were sensitive to *R. etli* CE3(pT2TFXK) as evidenced by zones of growth inhibition around the CE3(pT2TFXK) colonies. *A. vitis* growth was not inhibited by a non-TFX metabolite or nutrient competition by CE3(pT2TFXK) as evidenced by the lack of a zone around the near-isogenic *tfxA* mutant CE3(pT2TX3K) colony (Fig. 1). Wild type F2/5 produces a zone of inhibition versus the other agrobacteria used in this work. This occurred because of the antibiotic production previously observed by F2/5. These zones of inhibi-

Table 1: Bacterial Strains used in this work and their TFX phenotype (production, resistance, and sensitivity). Overproduction of TFX occurs when the TFX production is conferred using a multi-copy, broad host range plasmid such as pT2TFXK.

Strain	Characteristics	Reference
Rhizobium		
T24	*R. leguminosarum* bv. trifolii, TFX producing strain	28
T24::Tn5-1	TFX non-producing mutant, Tn5 insertion in *tfxB*	10
CE3(pT2TFXK)	*R. etli.* Contains TFX-encoding plasmid, overproduces TFX	20
CE3(pT2TX3K)	Plasmid contains *tfxA* deletion, non-TFX producing strain	20
ANU794	*R. leguminosarum* bv. trifolii, TFX-sensitive	29
ANU794(pT2TX3K)	TFX-resistant	This work
Agrobacterium vitis		
F2/5	Biological control on grapevine, no control on other host-plant species, TFX-resistant	30
F2/5(pT2TFXK)	Contains TFX-encoding plasmid, produces TFX, TFX-resistant	This work
F2/5(pT2TX3K)	Plasmid contains *tfxA* deletion, non-TFX producing strain, TFX-resistant	This work
CG49	Tumorigenic, nopaline-type Ti plasmid, controlled by F2/5, sensitive to TFX overproduction	31
CG78	Tumorigenic, vitopine-type Ti plasmid, not controlled by F2/5 co-inoculation, sensitive to TFX overproduction	from TJ Burr
K306	Tumorigenic, octopine-type Ti plasmid, controlled by F2/5, sensitive to TFX overproduction	from TJ Burr
CG107	Tumorigenic, sensitive to TFX overproduction	from TJ Burr
CG113	Tumorigenic, TFX-sensitive	from TJ Burr
CG435	Tumorigenic, TFX-sensitive	from TJ Burr

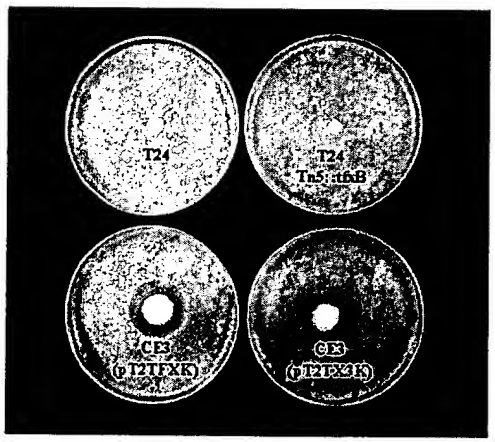

Figure 1
In vitro TFX antibiosis assay against A *vitis* CG78. Legend: The indicated TFX producing strains and their non-producing mutant derivatives cultured as a single colony near the centers of the plates and allowed to grow 4 days at 27°C. The plates were then misted with a dilute suspension of *A. vitis* CG78. The plates were photographed two days following misting. A zone of growth inhibition is present around the *R. etli* CE3(pT2TFXK) colony, but not around any of the other colonies.

tion were much larger when pT2TFXK was added to F2/5 and unchanged with the addition of pT2TX3K.

Evidence for TFX production by A. vitis strain F2/5(pT2TFXK)

Strain F2/5(pT2TFXK) inhibited TFX-sensitive R. leguminosarum bv. trifolii ANU794 but had no effect on ANU794 following addition of the TFX resistance genes provided by pT2TX3K (Fig. 2). Plasmids pT2TFXK and pT2TX3K confer resistance to TFX, tetracycline, and kanamycin with pT2TFXK also providing TFX production to a host strain [20]. Since strains F2/5 and F2/5(pT2TX3K) produced no zones of inhibition against ANU794 or ANU794(pT2TX3K) (data not shown), F2/5(pT2TFXK) is producing TFX. In addition, this shows that the agrocin-like molecule produced by F2/5 does not inhibit ANU794.

In planta biological control of crown gall conferred by TFX production

As expected, F2/5 did not inhibit galling by tumorigenic A. vitis strains on N. glauca. However, a 10:1 ratio of A. vitis F2/5(pT2TFXK):pathogen caused a statistically significant reduction in mean gall size relative to the TFX non-producing controls on N. glauca stems for all three tested tumorigenic strains (Figs. 3 and 4). High concentrations of F2/5(pT2TFXK) also reduced gall incidence for CG435 and CG78, but not for CG49 (Table 2). A 1:1 ratio of F2/5(pT2TFXK):CG435 also resulted in a significant reduction in gall size and in gall incidence compared to controls. A 1:1 ratio of F2/5(pT2TFXK):CG49 or F2/5

Table 2: Proportion of inoculations that resulted in gall formation by the tumorigenic *A. vitis* strains (CC49, CG78, and CG435) when co-inoculated with the TFX-producing strain F2/5(pT2TFXK) or the non-producing strains, F2/5 or F2/5(pT2TX3K).

	Tumorigenic Strain[a].	Proportion of inoculations that resulted in gall formation.	
Biological control	CG49	CG78	CG435
Strain			
F2/5	12/12	6/6	12/12
F2/5(pT2TFXK)	10/14	1/8	0/14
F2/5(pT2TX3K)	ND[b]	8/8	ND

a. Inoculations performed at 10:1 ratio of biological control:pathogenic strain. Presence of galls was scored visually by comparison to an uninoculated negative control one month post-inoculation. b. ND, not done.

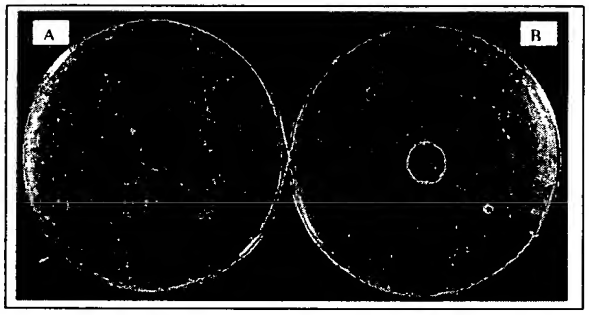

Figure 2
Ability of *Agrobacterium* to produce TFX with addition of pT2TFXK. Legend: Ability of F2/5(pT2TFXK) to inhibit ANU794. This inhibition is reversed by the addition of the TFX resistance genes to ANU794. Assay was performed as described in Figure 1 with F2/5(pT2TFXK) cultured in a single colony in the center of the plate. One day after growth at 28°C, the plates were sprayed with a dilute suspension of ANU794(pT2TX3K) (A) or ANU794 (B).

(pT2TFXK): CG78 did not affect either incidence of galls or reduce gall size. Similarly, an excess (a 1:10 ratio) of any of the virulent strains to F2/5(pT2TFXK) resulted in a high incidence of disease and large gall size.

Discussion

As all strains of *A. vitis* tested were sensitive to TFX both in this work and in a previous study [12], experiments were conducted to determine the effectiveness of a TFX-producing, avirulent strain of *A. vitis* in the prevention of crown gall caused a three strains of *A. vitis*. Although *A. vitis* F2/5 is an effective crown-gall biological control agent against most tumorigenic *A. vitis* strains when co-inoculated with a tumorigenic strain, it is not an effective against all strains of *A. vitis*.

Where *A. vitis* F2/5 is effective as a biological control agent, its control is only effective when numbers of F2/5 are equal to, or greater than, the number of cells of the virulent strain [3]. Strains that are resistant to F2/5 biological control are known. For example, strain CG78 is not controlled when co-inoculated with F2/5 [1].

Two principle benefits of TFX production by F2/5 are demonstrated here. Biological control is extended to a new host, *N. glauca*. Furthermore, biological control is extended to a strain that F2/5 fails to control (CG78). These effects are due to TFX production as demonstrated by the lack of efficacy of F2/5 against CG49, CG435, and CG78, and by the lack of efficacy of the near-isogenic TFX-non-producing F2/5(pT2TX3K) against CG78. TFX confers biological control when the TFX-producing strain is present in excess of the tumorigenic strain. Thus, F2/5(pT2TFXK) effectively inhibited gall incidence by two of the three tested tumorigenic strains when co-inoculated in approximately 10-fold excess. F2/5(pT2TFXK) inhibited gall size by more than 70% with third tumorigenic strain tested. At 1:1 or 1:10 inoculum ratios of F2/5(pT2TFXK):pathogen biological control was reduced or lost. High ratios of biological control: pathogen strain should be easily achieved in field situations by dipping the roots of planting stock in suspensions of the biological control strain, or by directly applying the bacterial suspension to the planting bed. Furthermore, TFX is inhibitory towards all tested species of *Agrobacterium*[12]. These results suggest that TFX production would enhance crown gall biological control for other biological control strains, such as *A. rhizogenes* K84, and on other host plants, especially where a mixed inoculum of different tumorigenic *Agrobacterium* species occurs.

K84 biological control is thought to be primarily due to production of two plasmid-encoded antibiotics, each of which accounts for a portion of the observed disease control [9]. This naturally occurring example suggests that

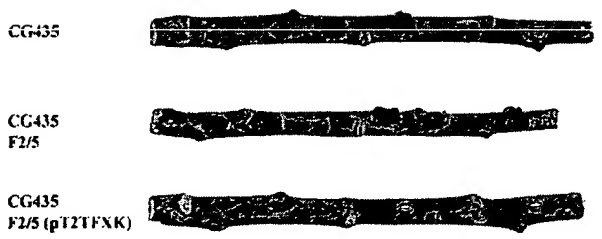

Figure 3
Effect of TFX production and inoculum ratio on gall formation. Legend: *Nicotiana glauca* stems were wounded with a dissecting needle, and 5 ul of inoculum (mixtures noted at left) was placed on the wounds. The top stem was inoculated with CG435 as a positive control. Stems 2 and 3 were inoculated with mixtures of biological control test strains and CG435 at either a 1:1 (left three inoculation sites on each stem) or 10:1 (right three inoculation sites) biological control:pathogen ratios. The photograph was taken approximately 2 months post-inoculation. pT2TFXK confers biological control on F2/5 at the 10:1 ratio, but not at the 1:1 ratio.

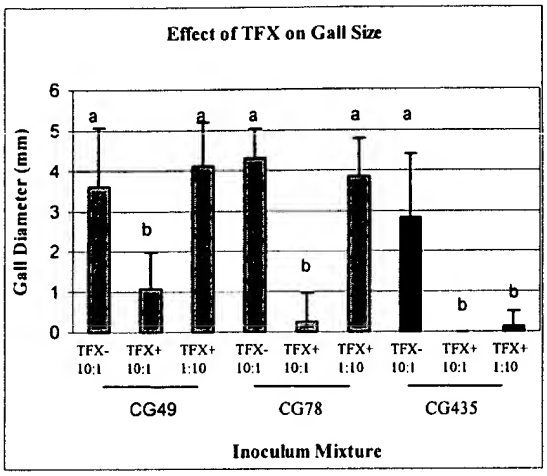

Figure 4
Effect of TFX production and inoculum ratio on *Nicotiana glauca* gall size. Legend: Gall diameter (mm) perpendicular to the stem was measured 1 month post-inoculation. Wound sites were inoculated with 5 µl of mixed bacterial suspensions. Mixtures were made immediately prior to inoculation. Each inoculum mixture was inoculated into three or four wounds on each of two plants, for a total of six to eight inoculations per treatment. Results that are significantly different at $\alpha = 0.05$ are indicated with different letters within a group of inoculum mixtures. High ratios of F2/5 (pT2TFXK) :tumorigenic strain result in significant disease suppression for all three tested tumorigenic strains.

pyramiding biological control mechanisms within one strain can enhance disease control. Strain F2/5 also produces an *A. vitis*-specific antibiotic *in vitro*, but this antibiotic is not involved in disease control [4]. Thus, the addition of TFX to F2/5 is a successful example of pyramiding unrelated disease control mechanisms and clearly demonstrates that individual antibiotics can be effectively developed as a mechanism of disease control.

Although plasmid-borne traits are frequently unstable, pT2TFXK contains the RK2 plasmid-partitioning locus that confers a high degree of stability both *in vitro* and under field conditions [15]. Stable TFX expression would be beneficial under field conditions when the biological control agent is inoculated on seeds or on roots dipped in a bacterial suspension prior to planting.

Trifolitoxin production enhances rhizosphere competitiveness of *Rhizobium etli* CE3 (pT2TFXK) in sterile soil and enhances nodulation efficiency in non-sterile soil when compared to a TFX-sensitive strain [20]. Trifolitoxin production also increases bean nodulation competitiveness of *Rhizobium etli* CE3 in the presence of indigenous rhizobia under agricultural conditions [15]. Field application of *R. etli* CE3 (pT2TFXK) dramatically reduced the diversity of indigenous α-proteobacteria in the bean rhizosphere without affecting unrelated species [13]. Thus, TFX production by *A. vitis* F2/5 should enhance the competitiveness and aid in establishment of this beneficial strain in the rhizosphere. The ability to displace indigenous tumorigenic *Agrobacterium* strains would be beneficial in those areas already infested with the pathogen.

Compared to other treatments available for pathogenic agrobacteria, the effects of TFX on non-target species is very low. Copper, a broad spectrum bacteriocide, is commonly used as a treatment of bacterial diseases. Although no comprehensive studies have been published on the taxonomic range of bacteria inhibited by K84, it is known that K84 produces multiple antibiotics and that K84 can inhibit strains of *Erwinia* and *Pseudomonas* that are unaffected by TFX [23-25]. TFX-producing strains inhibit only a very specific group within the α-proteobacteria in culture and in the field [13,26]. These observations suggest that a K84-producing strain may have a more serious im-

pact on non-target organisms than does a TFX-producing strain. However, there has been no study of the effects of K84 on non-target soil bacteria by culture-independent means to compare the effects of K84 versus the TFX system in a natural system.

Conclusions

TFX production effectively enhances crown gall biological control by *Agrobacterium vitis* F2/5. The host-plant range and range of tumorigenic *A. vitis* strains controlled by F2/5 are both broadened by TFX production. In addition, virulent strains of *A. vitis* not previously controlled by F2/5 are controlled following the addition of the trifolitoxin production and resistance genes to F2/5. The plasmid used to confer trifolitoxin production and resistance, pT2TFXK, is stably maintained in the absence of selection pressure [20]. This plasmid is not self-transmissible but is mobilizable. With the TFX system, the biological control of *Agrobacterium* pathogens may be extended to many crops far beyond what is possible today with currently available commercial products.

Materials and Methods

Bacterial and plant growth conditions, strain construction

Bacterial strains are listed in Table 1. The *A. vitis* strains (without pT2TFXK or pT2TX3K) were obtained from Dr. T.J. Burr, Cornell University. Bacteria were grown on BSM agar [21] media at 27°C. *A. vitis* F2/5(pT2TFXK) and F2/5(pT2TX3K) were constructed by triparental mating using standard procedures. Transconjugants were selected on BSM media amended with 50 μg/ml kanamycin. Trimethoprim (10 μg/ml) was added to counterselect the *E. coli* DH5α donor and helper strains. The helper strain was *E. coli* DH5a pRK2013 [27]. Strains containing the plasmids pT2TFXK and pT2TX3K were grown for routine propagation on BSM amended with 50 μg/ml kanamycin. Prior to use in making inoculum suspensions for biological control assays these strains were grown overnight on BSM agar without kanamycin.

Plants (*Nicotiana glauca*) were grown in the greenhouse with supplemental illumination and fertilized as needed with a nutrient solution called CNS containing (2 mM $CaCl_2.2H_2O$, 0.5 mM $MgSO_4.7H_2O$, 2 mM KCl, 0.4 mM KH_2PO_4, 2.5 mM NH_4NO_3, 0.065 mM $FeSO_4.7H_2O$, 2.3 μM H_3BO_3, 0.9 μM $MnSO_4.H_2O$, 0.6 μM $ZnSO_4.7H_2O$, 0.1 μM $NaMoO_4.2H_2O$, 0.11 μM $NiCl_2.6H_2O$, 0.01 μM $CoCl_2.6H_2O$, 0.15 μM $CuSO_4.5H_2O$).

In vitro antibiosis assay

TFX antibiosis assays were performed as described previously [11]. The effect of TFX on various *Agrobacterium vitis* strains (Table 1) was assessed using *Rhizobium leguminosarum* bv. trifolii strain T24 and *R. etli* strain CE3 (pT2TFXK) as producing strains. *Rhizobium leguminosarum* bv. trifolii T24::Tn5-1 and *R. etli* CE3 (pT2TX3K) were used as TFX non-producing negative controls.

In planta biological control of crown gall by TFX-producing strains

Agrobacterium vitis strains were suspended in sterile distilled water prior to the determination of colony forming units (CFU) per ml. These suspensions were adjusted to OD_{650} 0.10 (approximately 10^8 CFU/ml) using a Shimadzu UV-160 spectrophotometer and sterile distilled water, and stored until inoculation at 4°C. Actual inoculum viability and cell density were measured by dilution plating on BSM agar medium on the day that plants were inoculated [22]. Prior to inoculation, tumorigenic strains were diluted 10-fold with sterile distilled water to approximately 10^7 CFU/ml. Strain F2/5 and its derivatives were left undiluted, or diluted 10-fold (for CG49 and CG435 experiments) or 100-fold (for CG78 experiments). Thus, the CFU ratios were approximately 10:1, and 1:1 or 1:10 avirulent:tumorigenic strain. Immediately prior to plant inoculation tumorigenic strains were mixed 1:1 (vol:vol) with the appropriate biological control test strain. Positive controls were diluted 1:1 (vol:vol) with water. Thus, all plant inoculum contained approximately 5×10^6 CFU/ml of the tumorigenic strains. Plants (*Nicotiana glauca*) were inoculated by wounding the stem with a dissecting needle. Three or four inoculations were made per inoculum mixture on each of two plants. Thus, each of the two experiments included 6 to 8 repetitions per treatment. A 5 μl drop of bacterial suspension was placed on the wound and allowed to air dry. Inoculation sites were wrapped loosely with Parafilm (American National Can) for 1 week post-inoculation. Gall diameter perpendicular to the long axis of the stem was measured 4 to 7 weeks post-inoculation using a caliper, and all measurements were included for statistical analysis. Results were analyzed using ANOVA at the $\alpha = 0.05$ level of significance.

Abbreviations

TFX, trifolitoxin.

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USDA/ARS
North Dakota State University
Northern Crop Science Lab
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Fargo, ND 58105-5677
Telephone: 701/239-1337
Fax: 701/239-1369
E-mail: medwards@badlands.nodak.edu

Virology
Bradley I. Hillman
Rutgers University, Cook College
Plant Pathology Department
339 Foran Hall
New Brunswick, NJ 08903
Telephone: 908/932-9375x334
Fax: 908/932-9377
E-mail: hillman@aesop.rutgers.edu

Genetics and Population Biology of Fungi
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International Rice Research Institute
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E-mail: h.leung@cgnet.com

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Box 3BE
Las Cruces, NM 88003
Telephone: 505/646-4110
Fax: 505/646-8087
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Soilborne Fungi and Nematology
Jeanne Mihail
University of Missouri
Plant Pathology Department
108 Waters Hall
Columbia, MO 65211
Telephone: 573/882-1471
Fax: 573/882-0588
E-mail: mihail@psu.missouri.edu

Biological Control
David M. Weller
USDA/ARS
Washington State University
Plant Pathology Department
367 Johnson Hall
Pullman, WA 99164-6430
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Biological Control of Grape Crown Gall by Strain F2/5 Is Not Associated with Agrocin Production or Competition for Attachment Sites on Grape Cells

T. J. Burr, C. L. Reid, E. Tagliati, C. Bazzi, and S. Süle

First and second authors: Department of Plant Pathology, Cornell University, Geneva, NY 14456; third and fourth authors: Institute of Plant Pathology, University of Bologna, 40126 Bologna, Italy; and fifth author: Plant Protection Institute of Hungarian Academy of Sciences, P.O. Box 102, 1525 Budapest, Hungary.
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ABSTRACT

Burr, T. J., Reid, C. L., Tagliati, E., Bazzi, C., and Süle, S. 1997. Biological control of grape crown gall by strain F2/5 is not associated with agrocin production or competition for attachment sites on grape cells. Phytopathology 87:706-711.

Agrocin-minus mutants of nontumorigenic *Agrobacterium vitis* strain F2/5 controlled grape crown gall as well as the wild-type strain, indicating that agrocin is not a major factor in the mechanism of biological control. Relative levels of attachment to grape cells by tumorigenic and biocontrol strains were also measured. Attachment of tumorigenic strains (CG49 and K306) and biological control strains (F2/5 and agrocin-minus mutant 1077) was often reduced when mixtures of the strains were applied. However, high populations (10^3 to 10^5 CFU/ml) of all strains attached following mixed inoculations, suggesting that competition for attachment sites is also not a factor in the mechanism of biological control. Transfer of T-DNA to grape by CG49 was prevented or greatly inhibited in the presence of F2/5 or 1077 as measured by expression of the GUS reporter gene. The Ti plasmid virulence genes, however, were induced by exudates from grape shoots that had been inoculated with F2/5. Sonicated and autoclaved preparations of F2/5 and 1077 did not control crown gall or inhibit T-DNA transfer. Control by F2/5 is specific to grape, since gall formation on tomato, sunflower, and *Kalanchoe daigremontiana* were not inhibited.

Agrobacterium vitis (21), which causes crown gall disease on grape, survives endophytically in grape and, therefore, is disseminated in propagation material (2,15,29). Recently, methods for producing pathogen-free grapevines using shoot tip culture (3) and heat therapy (5) have been tested. However, once clean vines are obtained, it will be necessary to prevent them from becoming infected by *A. vitis* that may persist in decaying grape debris in soils (7).

A. radiobacter strain K-84 has been used successfully as a biological control of crown gall on several plant species (23). An agrocin produced by K-84 (agrocin 84) is thought to be a primary factor in the mechanism of control. K-84, however, is not effective for preventing infections on grape caused by *A. vitis* and, therefore, several laboratories have attempted to identify biological controls for grape crown gall (17,27,31,33). Xiaoying isolated a nontumorigenic strain of *A. tumefaciens* biovar 1 (HLB-2) that inhibited growth of several *A. vitis* strains and suppressed development of crown gall on grape in the greenhouse (8,33). Staphorst et al. (27) evaluated 16 strains, including strain F2/5 that inhibited growth of most *A. vitis* strains in vitro and greatly inhibited crown gall on grape in greenhouse experiments. Burr and Reid (6) reported that F2/5 produces an agrocin that is inhibitory to most *A. vitis* strains in vitro and F2/5 is effective for inhibiting tumor formation at wound sites on grape artificially inoculated with several *A. vitis* strains. F2/5 was most effective when wounds were inoculated at the same time as the pathogen or prior to the pathogen and at ratios of 1:1 (F2/5 to pathogen).

Prior to infection, tumorigenic *Agrobacterium* spp. attach to plant cells. It has been demonstrated that nontumorigenic strains of *Agrobacterium* may prevent infections by competing with tumorigenic strains for attachment sites on plant cells (23). The purpose of this research was to gain further insight into the mechanism by which F2/5 inhibits crown gall of grape. Specifically, we wished to determine if agrocin production, competition for attachment sites, or both are related to the mechanism of biological control. We also examined the ability of F2/5 to prevent transfer of T-DNA by tumorigenic strains to grape and to prevent the induction of Ti plasmid virulence (*vir*) genes. In addition, the specificity of F2/5 for preventing crown gall on plants other than grape was determined.

Corresponding author: T. J. Burr; E-mail address: tjb1@cornell.edu

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MATERIALS AND METHODS

Bacterial strains. Strains used are listed in Table 1. All strains were stored at −80°C in a medium containing (per 100 ml) 0.8 g of nutrient broth (Difco Laboratories, Detroit), 15 ml of glycerol, 0.2 g of yeast extract, and 0.5 g of glucose.

Transposon mutagenesis. F2/5 and *Escherichia coli* strain S17-1, carrying the transposon Tn5 on pSUP2021 (24), were grown for 48 h at 28°C on potato dextrose agar (PDA) (Difco Laboratories) and on Luria-Bertani medium (19) amended with kanamycin (50 μg/ml), respectively. Growth from two culture plates of each strain were suspended in 1.0 ml of sterile distilled water (SDW), and suspensions of the bacteria were combined. Volumes of 250 μl of the mixed suspension were spread onto the surface of four PDA plates and incubated at 28°C for 48 h. Resulting bacterial growth from the matings was scraped from the plates, suspended in SDW, and dilution-plated on AB minimal salts medium (9,32) containing kanamycin (50 μg/ml). Transconjugants that grew within 3 to 4 days were recultured on the same medium and then assayed for their ability to inhibit growth of *A. vitis* strain K306 in vitro.

In vitro antibiosis assays were done by first making suspensions of transconjugants containing about 10^8 CFU/ml (optical density of 0.1 at 600 nm [OD_{600}] determined with spectrophotometer). Twenty-four transconjugants were spotted (5-μl volumes) on each 9-cm-diameter petri dish containing about 10 ml of mannitol-glutamate (MG) medium (20). Plates were incubated for 48 h at

28°C, after which bacterial cells were killed by chloroform vapor (6), and bacterial growth was scraped from the plates. The surfaces of the plates were then sprayed until lightly wetted with a suspension of K306 containing about 10^8 CFU/ml. Plates were incubated at 28°C, and the presence of inhibition zones around transconjugants were observed after 48 h. Strain F2/5 was spotted on all plates as a positive control. Transconjugants that failed to inhibit growth of K306 were retested at least three times.

Southern hybridizations. Southern hybridizations were done on F2/5 and agrocin-minus mutant strains (1076, 1077, 1078, and 1079) to determine if the transposon had inserted into a plasmid or the bacterial chromosome. Plasmids and total genomic DNA were isolated from *A. vitis* strains as previously reported (4,7,25). Genomic DNA was digested overnight in *Eco*RV. Plasmids and digested genomic DNA were electrophoresed separately at 5 V/cm in 0.7% agarose in Tris-borate-EDTA and then visualized by staining with ethidium bromide. DNA was then Southern-transferred to nylon transfer membranes by alkaline transfer. Plasmids and digested total genomic DNA were hybridized with DNA from a pUT plasmid carrying mini-Tn*5Km2* (kanamycin-resistance gene) (12) that was labeled by random primed incorporation of digoxigenin-labeled deoxyuridine-triphosphate using a nonradioactive DNA labeling kit (Genius; Boehringer Mannheim Biochemicals; Indianapolis, IN).

Membranes were prehybridized in hybridization solution (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.5% [wt/vol] blocking reagent, 0.1% [wt/vol] *N*-lauroyl sarcosine sodium salt, and 0.02% [wt/vol] sodium dodecyl sulfate [SDS]) at 68°C for 135 min. Hybridizations were done at 68°C for 18 h in 0.03 ml of hybridization solution/cm^2 of membrane containing 25 ng of labeled, denatured DNA/ml of hybridization solution. The DNA probe was denatured for 10 min at 95°C and quick-chilled on ice for 2 to 5 min before it was added to the hybridization solution. Following posthybridization rinses, blocking, and incubation with antibody-conjugate solution and subsequent rinses, the membrane was placed in a hybridization bag that was open on two sides and saturated with a sufficient quantity of Lumi-Phos 530 (for chemiluminescent detection of alkaline phosphatase; Boehringer Mannheim Biochemicals) to fully wet the membrane. It was then incubated in the dark (covered with aluminum foil) for 1 min. The excess Lumi-Phos 530 was then drained from the hybridization bag, and the bag sealed, wrapped in foil, and incubated at 37°C for 30 min. The membrane was subsequently placed in a film cassette, exposed to X-ray film (Kodak XAR; Eastman Kodak Co., Rochester, NY) for 8 min, and then developed.

Gall inhibition assays. The ability of agrocin-minus mutants 1076, 1077, 1078, and 1079 to inhibit gall formation by *A. vitis* strains CG49 and K306 was compared with that of F2/5. Woody stems of potted 'Chardonnay' grapevines were inoculated using procedures that were previously reported (6). Briefly, wounds were made with an electric drill (6-mm diameter) and inoculated with 75 µl of bacterial cell suspensions containing approximately equal numbers of biological control and tumorigenic bacterial strains. Bacterial cell suspensions were made to OD_{600} 0.1, corresponding to about 10^8 CFU/ml, and populations were verified by plating on PDA or PDA amended with kanamycin. For each replication of the experiment, three inoculations were made to each of three plants, and inoculation sites were wrapped with Parafilm. The numbers of inoculation sites at which galls developed and the cross-sectional area (at widest point) of each gall were recorded 8 weeks after inoculation. SDW was applied as a negative control, and strains CG49 and K306 mixed with SDW as positive controls. The experiment was repeated four times. Differences in gall cross-sectional areas were analyzed using SAS General Linear Models *t* test (SAS Institute, Cary, NC).

Strains F2/5 and 1077 were also tested for their ability to inhibit gall formation by CG49 and K306 on sunflower (*Helianthus annuus* L.), tomato (*Lycopersicon esculentum* Mill.), and *Kalanchoe daigremontiana* Hamet & E. Perrier. Inoculation mixtures were made as above, and stems of potted plants were inoculated by placing 2-µl drops of strains, alone and in mixtures, at sites in which wounds were made with a sterile pin. The incidence of galls was recorded after 3 weeks. The experiment was repeated twice with K306 and once for CG49.

Attachment assays. Green, actively growing shoots were collected from potted grapevines (cv. Chardonnay). Sections of second, third, and fourth internodes having approximately the same diameters were cut from the shoots and surface-disinfested by shaking them submersed in 95% ethanol for 2 min and then in 10% Clorox for 5 min. They were then rinsed three times in SDW. The internodes were cut in lengths of about 1 cm, and the apical end was embedded in water agar in petri dishes (12 sections/plate). Bacterial strains CG49, F2/5, and 1077 were grown on PDA or PDA plus kanamycin for 48 h at 28°C, and then suspended in SDW to a concentration of OD_{600} 0.1. Serial dilutions of suspensions were plated. The exposed basal end of each shoot piece was then inoculated with 2 µl of the following treatments: SDW, CG49, F2/5, 1077, and CG49 combined with F2/5, and CG49 combined with 1077 (mixtures of strains containing equal volumes of each bacterial suspension). Twelve shoot sections were inoculated with each treatment. Lids were placed on the petri dishes, and internodes were incubated for 3 h. Another experiment was done substituting K306 for CG49. All experiments were repeated at least once.

The numbers of bacterial cells in each treatment that attached to the cut ends of the internodes were determined. The inoculated ends were excised (3- to 4-mm thickness), placed in tubes containing 10 ml of SDW, and vortexed vigorously for 30 s. Water was decanted from the tube, replaced with another 10 ml, and vortexed again. The vortexing in fresh water was done a total of three times. Three groups of four internode sections each were triturated in 1 ml of SDW in a microfuge tube, and populations of bacteria that had attached to the grape cells were determined by dilution-plating in triplicate on a medium, developed by Roy and Sasser, that is semiselective for *A. vitis* (RS) (22) and on PDA. Colonies were counted after 3 days on PDA and after 7 days on RS. CG49 could be distinguished from F2/5 and 1077 by colony morphology on PDA, and K306 could be distinguished from the other strains on RS.

Effect on T-DNA transfer. We wished to determine whether strains F2/5 and 1077 prevent the transfer of the Ti plasmid T-DNA to grape. CG49 was first transformed with p35SGUSINT (carries the *uidA* reporter gene and an eukaryotic intron driven by

TABLE 1. Bacterial strains used in this study

Designation	Characteristics	Origin or reference
Agrobacterium vitis		
CG49	Nopaline Ti plasmid	Burr, NY
K306	Octopine Ti plasmid	A. Kerr, Australia
CG49(p35SGUSINT)	Km^R	This study
K306(p35SGUSINT)	Km^R	This study
CG49(pSM243cd)	Km^R, Cb^R	This study
K306(pSM243cd)	Km^R, Cb^R	This study
F2/5	Agrocin$^+$ nontumorigenic	Staphorst et al., 1985 (South Africa)
1076	Agrocin$^-$ F2/5, Km^R	This study
1077	Agrocin$^-$ F2/5, Km^R	This study
1078	Agrocin$^-$ F2/5, Km^R	This study
1079	Agrocin$^-$ F2/5, Km^R	This study
Escherichia coli		
S17-1(pSUP2021)	Km^R	Simon et al., 1983
S17-1(p35SGUSINT)	Km^R	Vancanneyt et al., 1990
MC1061(pSM243cd)	Km^R, Cb^R	Stachel and Zambryski, 1986
HB101(pRK2013)	Km^R	Figurski and Helinski, 1979
Other		
A348(pSM243cd)	*A. tumefaciens* biovar 1	Stachel and Zambryski, 1986

the 35S promoter) (30). CG49 was grown on PDA and *E. coli* strain S17-1 (p35SGUSINT) was grown on LB medium amended with kanamycin. Transconjugants of CG49 carrying p35SGUSINT were derived by mating the *A. vitis* strains with S17-1 following the protocol described above for Tn5 mutagenesis and selecting transconjugants on minimal medium plus kanamycin.

CG49 and CG49(p35SGUSINT), alone and in combination with F2/5 and 1077, were inoculated on grape internodes (cvs. Cabernet Sauvignon and Catawba) as described above. Internodes were incubated in baby food jars containing half-strength Gamborg's B5 basal salt mixture (Sigma Chemical Co., St. Louis) amended with 10 g/liter of sucrose and 0.6% agar without hormones. Eight internodes were inoculated per jar, and two jars were used for each treatment.

To measure GUS activity, thin sections of the inoculated ends of the internodes were collected 14 days after inoculation. Sections were rinsed in 0.1 M phosphate buffer (pH 7.0) three times and then placed in individual wells of 96-well microtiter plates to which 100 µl of substrate solution was added. To make the substrate solution, a desired quantity of 5-bromo-4-chloro-3-indolyl-D-glucuronic acid (X-gluc) was first dissolved in a couple of drops of *N,N*-dimethyl formamide and then added at a concentration of 1 mg/ml of buffer consisting of 980 µl of 0.1 M phosphate buffer (pH 7.0) and 10 µl each of potassium ferricyanide and potassium ferricyanide (5 mM). Lids were placed on plates, wrapped in Parafilm, and the plant sections were incubated overnight at 37°C and then rinsed with phosphate buffer before viewing them under a dissecting scope for the presence of blue cells. After the first experiment showed that F2/5 and 1077 inhibited grape transformation, the experiment was repeated two additional times and included sonicated (in ultrasonicator for 60 s) and autoclaved preparations of F2/5 and 1077.

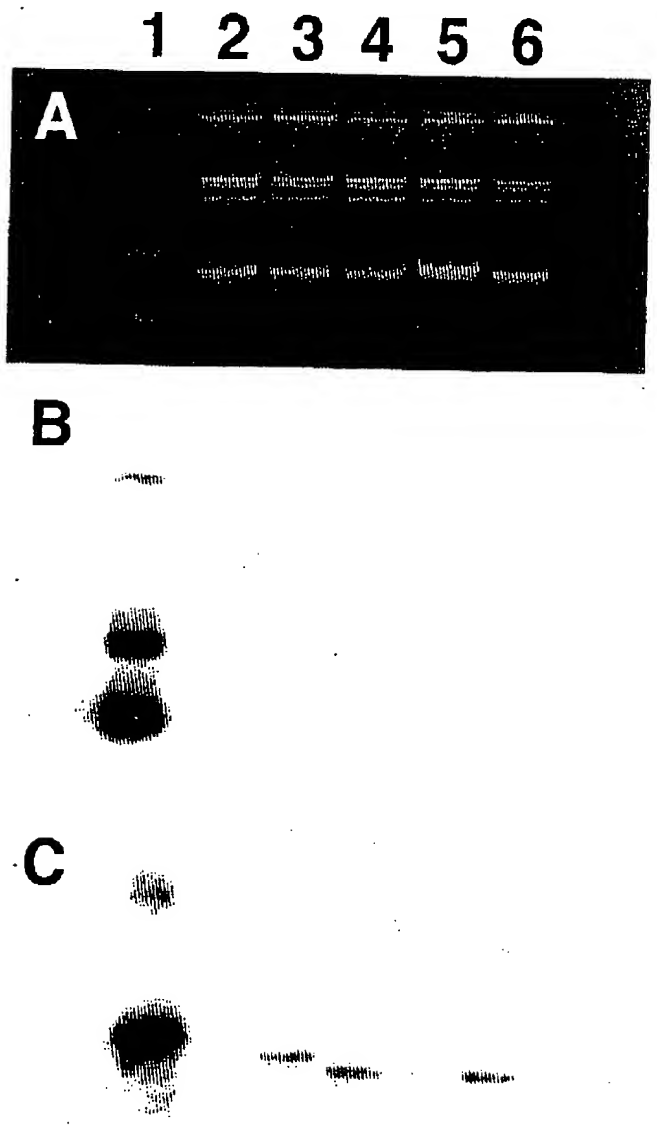

Fig. 1. A, Plasmid profiles; B, Southern hybridization of plasmids; and C, hybridization of *Eco*RV-digested total genomic DNA. All hybridizations were done by probing with mini-Tn*5Km2* as described in text. Lanes correspond to strains: 1 = S17-1(pSUP2021), 2 = F2/5, 3 = 1076, 4 = 1077, 5 = 1078, and 6 = 1079.

Effect of F2/5 on *vir* induction. A previously employed assay (28) was used to determine if the induction of *vir* in CG49 and K306 is inhibited by exudates from grape shoots that were inoculated with F2/5 and 1077. Transconjugants of CG49 and K306 carrying pSM243cd (containing a *virB::lacZ* fusion) (26) were made by triparental-mating with *E. coli* strain MC1061 carrying pSM243cd and with *E. coli* strain HB101 carrying the helper plasmid pRK2013 (13).

In vitro-grown grape shoot pieces (*Vitis vinifera* cv. Narancsizü), about 10 mm in length, were submersed for 20 min in suspensions of F2/5 or 1077 containing about 10^8 CFU/ml. Control shoot pieces were submersed in SDW. CG49(pSM243cd), K306(pSM243cd), and *A. tumefaciens* biovar 1 strain A348(pSM243cd) (as a positive control) were grown for 48 h on low-phosphate AB sucrose medium, which is optimized for *vir* induction assays (32). The medium contains 3% sucrose, AB salts, 2.5 mM phosphate buffer, 20 mM morpholineethanesulfonic acid (MES), and 50 µg/ml each of ampicillin and kanamycin. The medium was adjusted to pH 5.5 for *vir* induction. The bacteria were then suspended in the same liquid medium (to about 10^8 CFU/ml) without antibiotics. They were then spread on the surface of the above medium containing 1% agar, 20 mg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and 20 mg of IPTG (isopropyl-β-D-thiogalactopyranoside)/liter. The grape shoot pieces were placed on the surface of the medium, and the appearance of blue color around the cut ends of the shoots (denoting production of β-galactosidase and, thus, *vir* induction) was monitored 2 to 4 days later.

TABLE 2. Effect of F2/5 and 1077 on attachment of CG49 and K306 to grape[a]

Coinoculant	Mean population[b]		
	CG49/K306[c]	F2/5	1077
CG49			
Water	1.1×10^5 u[d]	1.5×10^5 w	7.2×10^4 y
CG49	...	5.7×10^4 x	3.0×10^4 z
F2/5	4.1×10^4 v	...	...
1077	3.2×10^4 v	...	...
K306			
Water	6.5×10^3 u	6.3×10^3 w	6.1×10^3 y
K306	...	8.0×10^3 w	3.2×10^3 z
F2/5	6.7×10^3 u	...	...
1077	4.8×10^3 u	...	...

[a] Initial bacterial suspensions (CFU/ml) used with CG49 were CG49 = 1.0×10^8, F2/5 = 1.3×10^8, and 1077 = 9.0×10^7; and those with K306 were K306 = 7.0×10^7, F2/5 = 4.0×10^7, and 1077 = 5.0×10^7 mixed with equal volumes of water or coinoculant and applied to grape shoots as described in text.

[b] Mean populations (CFU/ml) of strains that attached to grape shoots following inoculations with other strains were determined by plating on potato dextrose agar (PDA) or RS media. Colonies of CG49 were distinguished from F2/5 and 1077 by morphology on PDA. Colonies of K306 were distinguished from colonies of other strains on RS medium.

[c] Mean populations of CG49 for the first half of the table and of K306 for the second half.

[d] Values in the same column that are followed by different letters are significantly different determined by the SAS General Linear Models *t* test ($P = 0.05$).

RESULTS

Agrocin-minus F2/5 mutants. About 3,000 Tn5 mutants of F2/5 were screened for their capacity to inhibit growth of K306 in vitro. Four agrocin-minus mutants, 1076, 1077, 1078, and 1079, were isolated. Plasmid profiles of the mutant strains (Fig. 1A) revealed identical patterns to F2/5, and none of the plasmids hybridized with the mini-Tn5*Km2* probe (Fig. 1B). In all cases, digested genomic DNA from mutant strains revealed a single band of approximately the same size when hybridized with the Tn5*Km2* probe (Fig. 1C).

To determine if the mutations may not be affecting agrocin production but rather the export of agrocin across the outer membrane of the bacterial cell, strains F2/5 and 1077 were grown on MG medium and then an aqueous suspension of about 10^8 CFU/ml was ultrasonicated for 3 min and spotted on MG medium. After the spots dried, the plates were sprayed with a suspension of K306 as described above. Zones of growth inhibition were only observed around the F2/5 spots, indicating that mutants were unable to produce the agrocin.

Gall inhibition. Agrocin-minus derivatives 1076, 1077, 1078, and 1079 controlled crown gall on grape as well as did wild-type F2/5 (Fig. 2). Galls did not develop, or were greatly suppressed, on plants that were coinoculated with the F2/5 or mutants mixed with CG49 or K306. As observed previously, K306 is more virulent on grape than is CG49, in that more inoculation sites develop galls and they are generally larger in size (6).

Strains F2/5 and 1077, however, failed to inhibit gall formation by CG49 and K306 on sunflower, tomato, or *K. daigremontiana*. All plants that were coinoculated with the biological controls and pathogens developed galls at all inoculation sites, and the galls appeared identical to those that developed on plants inoculated with the pathogens alone.

Attachment. Following coinoculation with F2/5 and 1077, populations of CG49 that attached to grape shoot tissues were reduced significantly, whereas populations of K306 were not (Table 2). Populations of F2/5 and 1077 that attached to the shoots also were reduced significantly when coinoculated with CG49. Strain 1077 attachment was reduced significantly when coinoculated with K306. Although the reductions in attachment were, in some cases, statistically significant, they were no greater than one log unit as compared with attachment in the absence of coinoculant strains. Therefore, high numbers of CG49 and K306 cells still attached to grape, even in the presence of F2/5 and 1077. It was demonstrated that the populations of CG49 and K306 that were able to attach to the grape shoots in the presence of F2/5 and 1077 (about 10^4 CFU/ml) were adequate to cause crown gall on shoot pieces (T. J. Burr, unpublished data). Therefore, the mechanism by which F2/5 inhibits crown gall of grape does not appear to be associated with competition for attachment sites on grape cells.

T-DNA transfer and *vir* induction. Strains F2/5 and 1077 greatly affected the ability of tumorigenic strains to transfer T-DNA to grapes as determined by the presence of blue cells (GUS expression) in inoculated internode sections. A total of five to six thin sections from each of 16 internodes were observed for each inoculation made to 'Cabernet Sauvignon' and 'Catawba'. Internodes that were inoculated with wild-type CG49 had no blue cells, whereas those inoculated with CG49(p35SGUSINT) had abundant blue cells at the inoculated ends. In contrast, no blue cells were observed in tissues that were inoculated with the mixture of CG49(p35SGUSINT) and F2/5 or with CG49(p35SGUSINT) and 1077. In some cases, these shoots developed slight swellings that possibly resulted from normal callus formation, however, none had observable blue cells. Internodes that were inoculated with CG49(p35SGUSINT) mixed with sonicated or autoclaved F2/5 and 1077 had abundant blue cells in all internodes, resembling those inoculated with CG49(p35SGUSINT) alone. No differences were observed between experiments done with 'Cabernet Sauvignon' or 'Catawba'.

Treating grape shoot pieces with F2/5 or 1077 did not prevent their exudates from inducing *vir* in strains CG49 or K306. Four transconjugants of CG49 and two of K306 carrying pSM243cd were compared with the positive control, A348(pSM243cd). Blue-pigmented bacterial growth of CG49(pSM243cd) and K306(pSM243cd) was observed within 48 to 72 h on the agar plates around the cut ends of the shoots that were treated with F2/5, 1077, or water (indicative of β-galactosidase activity).

DISCUSSION

Two possible mechanisms by which F2/5 may inhibit crown gall of grape, i.e., antibiosis by an agrocin and competition for attachment sites on grape, were investigated. Although F2/5 produces an agrocin that inhibits growth of many *A. vitis* strains in vitro, the agrocin is apparently not a major factor in suppressing crown gall on grape, since agrocin-minus mutants of F2/5 provided the same level of crown gall control as the wild-type strain. This finding is particularly interesting since F2/5 was first selected as a potential candidate for biological control because of its in vitro antibiosis to *A. vitis* (27). We previously found that at least one strain of *A. vitis* (CG78) that is sensitive to the agrocin of F2/5 in vitro is not controlled by F2/5 on grape (6). Therefore, the mechanism by which F2/5 inhibits crown gall on grape is apparently not related to agrocin production and is different from that by which *A. radiobacter* strain K-84 controls crown gall on other hosts. Production of agrocin 84 is generally believed to be a major contributing factor to biological control by K-84, although other factors such as competition for attachment sites on plant cells (11) and the production of agrocins other than agrocin 84 (10) may also be involved.

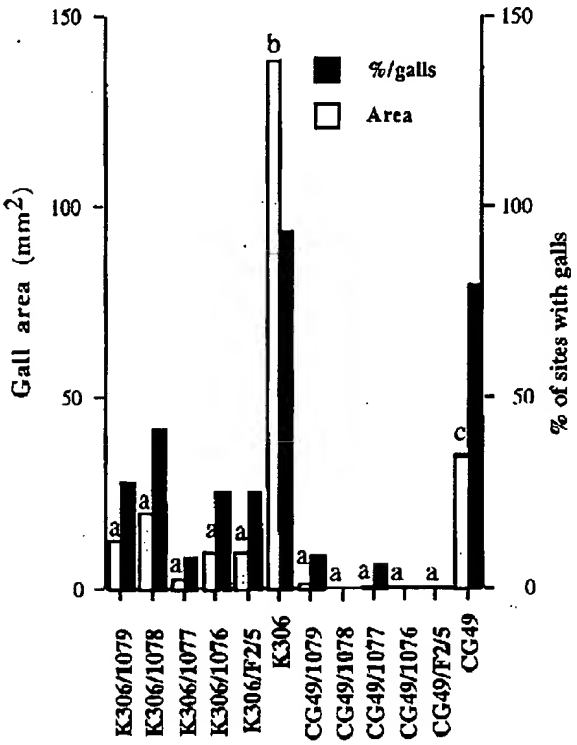

Fig. 2. Effect of F2/5 and agrocin-minus mutants 1076, 1077, 1078, and 1079 on the development of crown gall of grape by CG49 and K306. Suspensions of bacteria containing about 10^8 CFU/ml of CG49 or K306 were mixed 1:1 with water, F2/5, or agrocin-minus mutants before inoculating woody stems of grape as described in text. Values for gall size in columns followed by different letters differ significantly as determined by the SAS General Linear Models *t* test ($P = 0.05$).

An early event in tumorigenesis of *Agrobacterium* spp. is the attachment of the bacterium to host plant cells (16). At least three chromosomal virulence genes are known to be involved in attachment of *A. tumefaciens* to various plant species. It has been demonstrated that some nontumorigenic strains efficiently compete for attachment sites on plant cells and, thereby, inhibit attachment and infection by tumorigenic strains (18). It was previously demonstrated that *A. vitis* attaches more efficiently than *A. tumefaciens* to grape roots and that polygalacturonase production by *A. vitis* may be associated with attachment (1). Therefore, we examined the relative abilities of F2/5, 1077, CG49, and K306, alone and in combinations, to attach to grape cells. Our results do not support the hypothesis that F2/5 is preventing crown gall by significantly inhibiting the attachment of tumorigenic *A. vitis* to grape. Although there was a significant reduction in attachment of the tumorigenic strains following inoculations with some strain mixtures, this seemed to be a nonspecific phenomenon, since even attachment of biological control strains F2/5 and 1077 was reduced when they were inoculated together with CG49.

The effectiveness of F2/5 and agrocin-minus mutants for crown gall control was measured by observing gall development on plants and determining GUS expression in plants inoculated with derivatives of CG49 and K306 carrying p35GUSINT. Gall formation and GUS expression were often completely prevented, indicating that the T-DNA of the pathogens is not transferred to grape and expressed in the grape genome. In contrast, galls formed on sunflower, tomato, and *K. daigremontiana* that were inoculated with F2/5-pathogen mixtures, illustrating that CG49 and K306 remain viable (6) and tumorigenic in the bacterial mixtures. These results suggest that F2/5 may be inducing a resistance reaction in grape or that its inhibitory activity on the pathogen is directly related to its interaction with grape.

Plant signal compounds are able to induce the *vir* genes that are carried on the Ti plasmids of tumorigenic *Agrobacterium* spp. (16). These genes are responsible for packaging and transfer of T-DNA to the plant. We sought to determine if, following treatment of grape shoots with F2/5 and 1077, exudates from the inoculated shoots would be altered in their ability to induce *vir* in *A. vitis*. However, in all cases, *vir* was induced by exudates from grape shoots that had been treated with both strains. *Vir* induction was determined by measuring induction of a *virB::lacZ* fusion carried in pSM243cd. The *virB* was cloned from *A. tumefaciens* strain A6. It has been reported that some *vir* regions of *A. vitis* Ti plasmids may differ substantially from those that have been mapped in *A. tumefaciens* strains (14). Therefore, it was interesting to note that *vir* products from wild-type CG49 and K306 were able to induce the A6 *virB* in pSM243cd. Using this same method to measure differences in *vir* induction by exudates from crown gall-susceptible and -resistant grape genotypes, it was found that *vir* is induced equally well in both groups (28). Therefore, in crown gall-susceptible grape shoots treated with F2/5 and in resistant grape genotypes, the prevention of T-DNA expression in the plants does not result from failure of *vir* induction.

Biological control of grape crown gall is likely to provide an effective means for preventing infection of vines that are to be planted in vineyards where grapes were grown previously. An understanding of the mechanism of control will be important for determining the most efficient way of preparing and applying the biological control strains in commercial agriculture. Ideally, F2/5 applied to vines at planting time would colonize them systemically, similar to tumorigenic *A. vitis*, and provide protection against crown gall for an extended period of time. Several factors including the timing of F2/5 applications in relation to infection by *A. vitis*, the length of the protection period after application of F2/5, and the possible need for repeated applications of F2/5 during the life of the vineyard are being studied.

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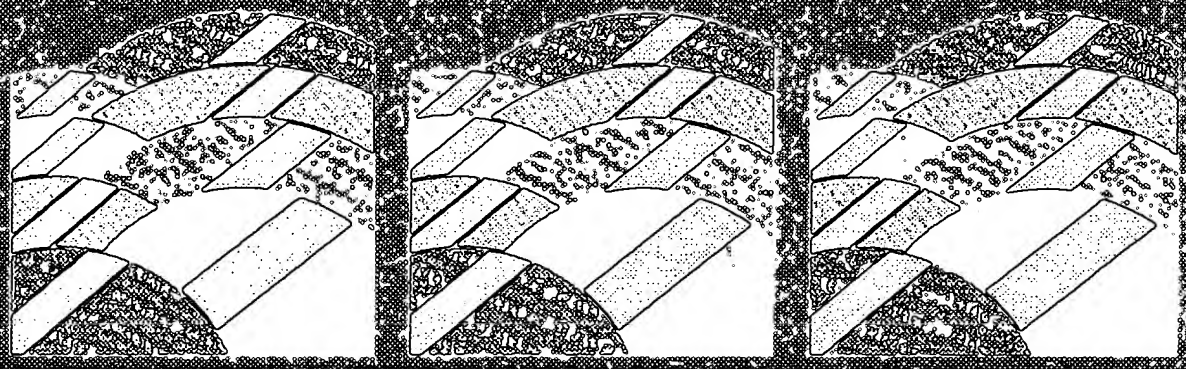

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Effects of antibiotic-producing *Streptomyces* on nodulation and leaf spot in alfalfa

Deborah A. Samac[a,b,*], Amy M. Willert[c], Matthew J. McBride[b], Linda L. Kinkel[b]

[a] *USDA-ARS-Plant Science Research Unit, 495 Borlaug Hall, 1991 Upper Buford Circle, St. Paul, MN 55108, USA*
[b] *Department of Plant Pathology, University of Minnesota, St. Paul, MN, USA*
[c] *Department of Biology, Hamline University, St. Paul, MN, USA*

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Abstract

The ability of antibiotic-producing streptomycetes to colonize alfalfa (*Medicago sativa* L.) plants and influence the activities of a fungal plant pathogen (*Phoma medicaginis* var. *medicaginis*) and a mutualistic symbiont (*Sinorhizobium meliloti*) was investigated. *Streptomyces* strains were introduced around seeds at the time of planting. Hyphal filaments and spore chains were observed by scanning electron microscopy on roots of alfalfa seedlings receiving the streptomycete amendment. *Streptomyces* strain densities on leaves decreased 10–100-fold over an 8-week period, while densities on roots remained constant over time. The *Streptomyces* strains also colonized alfalfa root nodules. We then tested the ability of 15 antibiotic-producing strains of *Streptomyces* to inhibit in vitro growth of *Phoma medicaginis* var. *medicaginis* Malbr. & Roum., the causal agent of spring blackstem and leaf spot of alfalfa. The majority of the *Streptomyces* strains inhibited growth of three diverse strains of *P. medicaginis*. In a detached leaf assay, one *Streptomyces* strain decreased leaf spot symptoms caused by *P. medicaginis* when inoculated onto leaves 24 h before the pathogen. Two *Streptomyces* strains decreased defoliation caused by *P. medicaginis* when the streptomycetes were introduced around seeds at the time of planting. We also examined inhibitory activity of *Streptomyces* strains against 11 strains of *S. meliloti*. Eight of the 15 *Streptomyces* strains inhibited in vitro growth of five or more of the *S. meliloti* strains, while four *Streptomyces* strains had no effect on growth of any test strains. In a growth chamber assay, two of six *Streptomyces* strains, when inoculated into the planting mix, significantly reduced plant dry weight compared to the treatment with *S. meliloti* alone, but did not significantly reduce the number of nodules. These results suggest that careful selection of *Streptomyces* isolates for use in biological control of plant diseases will limit the potential negative impacts on rhizobia.
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Keywords: Biocontrol; Lucerne

1. Introduction

Alfalfa (*Medicago sativa* L.) is the primary cultivated forage crop in the United States. Growth of alfalfa increases soil fertility, improves soil structure, and reduces erosion, making alfalfa an important component in crop rotations and in sustainable agricultural systems. However, a number of serious diseases affect persistence and yield of alfalfa. In particular, root rot and crown rot diseases, caused by a complex of fungi, as well as several foliar pathogens, can cause significant yield reductions. *Phoma medicaginis* var.

* Corresponding author. Tel.: +1-612-625-1243; fax: +1-651-649-5058.
E-mail address: dasamac@tc.umn.edu (D.A. Samac).

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medicaginis Malbr. & Roum., the causal agent of spring blackstem and leaf spot of alfalfa, infects leaves and stems during cool wet weather (Leath et al., 1988). The disease causes the most severe yield reduction in the first spring harvest, which typically has the highest dry matter production and forage quality. Infection of crowns and roots can cause stand thinning (Leath et al., 1988; Rodriguez et al., 1990), decreasing the productive life of an alfalfa field. Little resistance to *P. medicaginis* has been found in alfalfa germplasm and no highly resistant varieties are currently available.

We have investigated the use of *Streptomyces* spp. as broad-spectrum biological control agents for multiple pathogens in diverse cropping systems including potato, alfalfa, soybean, and corn. Streptomycetes are common filamentous bacteria that are effective, persistent soil saprophytes and often are associated with plant roots. They are well-known producers of antibiotics and extracellular hydrolytic enzymes. These characteristics, as well as their ability to withstand desiccation and high temperatures as spores, make them attractive as biological control agents. Several strains are available as commercial products (Nemec et al., 1996).

A naturally-occurring potato scab suppressive soil was found to have high population densities of antibiotic-producing, non-pathogenic streptomycetes (Lorang et al., 1995; Liu et al., 1996). These antibiotic-producing strains have strong in vitro inhibitory activity against a wide variety of plant-pathogenic bacteria and fungi (Liu, 1992; Jones and Samac, 1996; Xiao et al., 2002). Inoculation of soil with individual strains controls potato scab in greenhouse and field experiments (Liu et al., 1995). Individual strains control root rot caused by *Aphanomyces euteiches* Dresh. and *Phytophthora medicaginis* Hansen et Maxwell on alfalfa in growth chamber and greenhouse studies (Jones and Samac, 1996; Xiao et al., 2002) and reduce population densities of the root-lesion nematode (*Pratylenchus penetrans* (Cobb) Filipjev & Schuurmans Stekhoven) on alfalfa roots (Samac and Kinkel, 2001). Field experiments have shown control of Septoria leaf spot of hybrid poplar by foliar applications of single *Streptomyces* strains originating from the suppressive soil (Gyenis, 2000; Shimizu, 1994).

Streptomyces spp. have the potential to contribute to management of soilborne and foliar plant pathogens on diverse plant hosts. However, their value in a multiple crop, integrated disease management system depends not only on their abilities to control plant disease, but also on their ability to colonize plant host surfaces and their potential inhibitory effects on beneficial microbes. In particular, because of their production of broad-spectrum antimicrobial compounds, *Streptomyces* spp. may have negative impacts on the growth and performance of nitrogen-fixing mutualistic bacteria.

The objectives of this study were to evaluate the potential for *Streptomyces* spp. to contribute to the management of foliar and soilborne plant pathogens of alfalfa. Specifically, we investigated the ability of *Streptomyces* strains to colonize alfalfa leaves and roots following inoculation of the soil at planting. We also examined the ability of *Streptomyces* strains to inhibit *Phoma medicaginis* growth in vitro and quantified the effects of *Streptomyces* strains on leaf spot symptoms caused by *P. medicaginis*. Finally, we evaluated the effects of antibiotic-producing *Streptomyces* strains on in vitro growth of *Sinorhizobium meliloti* and nodulation of alfalfa plants.

2. Materials and methods

2.1. Bacterial and fungal strains

Streptomyces strains were isolated from a potato scab-suppressive soil in Grand Rapids, MN as described previously (Liu et al., 1996). Fifteen strains were selected for study here based on their ability to produce large zones (>10 mm diameter) of growth inhibition in vitro of *Streptomyces scabies*, the causal agent of potato scab (Liu et al., 1996). A subset of 11 strains from the USDA *S. meliloti* strain collection was obtained from M. Sadowsky, University of Minnesota. *Streptomyces* and *Sinorhizobium* strains were stored until use in 20% glycerol at −80 °C. *Phoma medicaginis* strain 866 was obtained from K. Leath, (USDA-ARS, University Park, PA), strain T430 from N. O'Neill (USDA-ARS, Beltsville, MD), and strain NY001 from G. Bergstrom (Cornell University, Ithaca, NY). Fungi were stored on silica gel at 4 °C.

Fresh spore suspensions from *Streptomyces* strains were collected in sterile distilled water with 0.01% Tween 20 from cultures grown for 7–10 days at 28 °C on oatmeal (OM) agar plates (Liu et al., 1995).

Inoculum concentrations were determined by comparing the optical density of the spore suspension at 600 nm with colony forming units (CFU) previously determined by dilution plating of the spore suspension on OM agar plates. *P. medicaginis* cultures were grown on potato dextrose agar (PDA) at 25 °C for 14–21 days for production of conidia. Fresh spore suspensions were collected in sterile distilled water with 0.01% Tween 20 and spore concentrations were determined using a hemacytometer. *S. meliloti* strains were cultured on YEM agar plates (Vincent, 1970) at 28 °C for 2 days and cell suspensions were made by vortexing colonies removed from plates in phosphate buffered saline. Inoculum concentrations were determined by comparing the optical density at 600 nm with CFU previously determined by dilution plating on YEM agar plates.

2.2. Alfalfa colonization

Seeds of the alfalfa variety Agate were surface sterilized by immersion in 70% ethanol for 1 min, followed by continuous agitation in a 10% bleach solution (0.525% sodium hypochlorite) for 10 min, and three rinses with sterile distilled water. Seeds were allowed to imbibe water overnight at room temperature. Plants were grown in 3.8 cm × 19 cm containers (Stuewe & Sons Inc., Corvallis, OR, USA) in a sterilized sand:vermiculite mixture (1:1 (v/v)). A fresh suspension of *Streptomyces* spores (strains 93, GS6-17, and GS43-11) in OM broth with 0.01% Tween-20 was added to the planting mixture immediately before planting so that each *Streptomyces*-amended container received approximately 1×10^6 CFU/cm^3 planting mix. Control treatments received an equal volume of OM broth. Seeds were placed on the surface of the planting mix approximately 2 cm from the top of the container and covered with a 0.5 cm layer of the sterilized sand:vermiculite mixture. Each plant was inoculated with approximately 1×10^4 CFU of *S. meliloti* strain USDA 105F21 in phosphate buffered saline 7 days after planting by pipetting the inoculum onto the soil mix at the base of the plant.

Plants were maintained in a growth chamber with a 16 h day length at 21 °C, approximately 600 μmol m^2/s irradiance, and night temperature of 19 °C. Plants were fertilized weekly with a nitrogen-free nutrient solution (Somasegaran and Hoben, 1994) and watered as needed. Alfalfa tissue samples were harvested for assessment of *Streptomyces* population densities at 3, 6, and 8 weeks after planting. The oldest trifoliolate leaf was removed and roots were then removed from the planting mixture, gently rinsed with distilled water to remove soil particles, and blotted dry. Roots were divided into the upper 5 cm root segment and the remaining lower root system. Nodules were removed from roots at 6 and 8 weeks after planting. Shoot, root, and nodule fresh weights were recorded. The trifoliolate leaf and root sections were sonicated separately for 15 min in 9 ml sterile distilled water with 0.01% Triton X-100 using a Bransonic 8200 water bath sonicator (Branson Ultrasonics Corp., Danbury, CT, USA). Nodules were homogenized in 9 ml sterile distilled water with 0.01% Triton X-100 using a Tissue Tearor (Research Products International Corp., Mount Prospect, IL, USA). Serial dilutions of wash suspensions and homogenates were plated on OM agar containing antibiotics (Loria and Devis, 1988). Streptomycete colony counts were recorded after incubation for 7 days at 28 °C. Five plants per treatment were analyzed, at each time point, and the experiment was repeated twice.

2.3. Scanning electron microscopy

Alfalfa roots from 14-day-old plants grown as described above and inoculated with *Streptomyces* strain 93 at planting were cut into 1 cm sections and fixed overnight in 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7. Roots were rinsed three times with 0.1 M phosphate buffer, pH 7.0 and stained for 30 min in 2% osmium tetroxide. After rinsing three times with distilled water, roots were dehydrated in a graded acetone series, then critical-point dried. Samples were mounted on aluminum pin stubs and coated with Au/Pd in a vacuum evaporator. Samples were visualized using a Philips SEM 500.

2.4. In vitro antibiosis assays

A double layer agar method (Vidaver et al., 1972) was used to determine in vitro inhibition of 3 strains of *P. medicaginis* and 11 strains of *S. meliloti* by each of the 15 *Streptomyces* strains. In order to determine the potential of *Streptomyces* strains to influence

the production of antimicrobial compounds by other *Streptomyces* strains in culture, antibiosis assays were carried out initially with one *Streptomyces* strain per agar plate and results were compared with assays in which four strains were cultured on each agar plate. For assays with one isolate per plate, 10 μl of a fresh spore suspension of each *Streptomyces* strain at 10^7–10^8 CFU/ml in sterile water with 0.01% Tween-20 was dotted onto the surface of 10 ml R2YE agar (Hopwood et al., 1985) in a 15 mm × 60 mm Petri-plate. Plates were incubated at 28 °C for 3 days. The *Streptomyces* cultures were killed by inverting cultures over watch glasses containing chloroform in a fume hood for 1 h. Traces of the solvent were removed by placing plates in a laminar flow hood for 30 min. Plates were then overlaid with 3 ml of molten 1% water agar at approximately 55 °C containing either *P. medicaginis* (approximately 10^6 spores/ml) or *S. meliloti* (approximately 10^7 CFU/ml). Each *Streptomyces–Phoma* or *Streptomyces–Sinorhizobium* combination was tested on three individual plates. Clear growth inhibition zones were measured after incubation at 28 °C for 3 days (*S. meliloti*) or 7 days (*P. medicaginis*). The same procedure was used in assays examining four strains on each agar plate, using 35 ml of R2YE medium in 100 mm × 15 mm Petri-plates. The plates were overlaid with 10 ml of molten water agar inoculated with *P. medicaginis* or *S. meliloti* as above. Each *Streptomyces–Phoma* or *Streptomyces–Sinorhizobium* combination was tested on three individual plates with variable combinations of *Streptomyces* strains.

2.5. Disease assays

A single plant from the alfalfa variety Regen-SY (Bingham, 1991) was vegetatively propagated and plants were grown in a growth chamber under the same conditions as described above. Leaves were removed from the oldest three nodes of several clones and placed abaxial side down on sterile moistened filter paper in covered plastic 100 mm × 15 mm Petri-plates, four leaves per plate. Leaves in each plate were sprayed with approximately 1 ml of a 1×10^8 CFU/ml suspension of *Streptomyces* spores in OM broth. Plates were incubated at room temperature for 24 h during which time the free moisture in the *Streptomyces* inoculum dissipated. Leaves in each moist chamber were then sprayed with approximately 1 ml of *P. medicaginis* strain 866 spores at 1×10^6 spores/ml. Control treatments of oatmeal broth followed by *P. medicaginis* inoculation and oatmeal broth followed by spraying with water were included in each experiment. Leaves were incubated at room temperature for 5–6 days; water was added periodically to each chamber to keep filter papers moist. Leaves were scored for leaf spot symptoms, where 0: no symptoms; 0.5:1–10 "tar spots" on each leaflet, no yellowing; 1: 10–15 spots on each leaflet, approximately 25% of leaf yellow, 2: more than 15 spots on each leaflet, approximately 50% of leaf yellow; 3: 75% of leaf yellow, 4: 100% of leaf yellow. For each *Streptomyces* strain, four plates were assayed with leaves within plates treated as subsamples. The experiment was carried out twice.

Eight-week-old plants inoculated with *Streptomyces* strains 93, GS6-17 or GS43-11 and *S. meliloti* USDA105F21 as in Section 2.2 (40 plants per treatment), were sprayed until run-off with a fresh spore suspension of *P. medicaginis* strain 866 at 1×10^6 spores/ml in sterile distilled water with 0.01% Tween-20. Plants were maintained in 100% relative humidity in darkness for 48 h then returned to the growth chamber. Defoliation of the primary stem of each plant was evaluated 7 days after inoculation.

2.6. Nodulation assay

Seeds of the alfalfa variety Agate were surface sterilized and imbibed on sterile filter paper moistened with sterile distilled water at room temperature overnight. Growth containers were assembled from two nested 110 mm Magenta boxes (Magenta Corporation, Chicago, IL, USA). The top Magenta box was modified with a 0.5 cm diameter hole in the base fitted with a rope wick into the bottom box. Approximately 340 cm^3 of a sand:vermiculite (1:1 (v/v)) mixture was placed into the top Magenta box, moistened with 200 ml of a nitrogen-free nutrient solution (Somasegaran and Hoben, 1994), and the containers autoclaved for 30 min on 2 consecutive days. A fresh *Streptomyces* spore suspension (approximately 6.8×10^7 CFU/ml) was added to the planting mixture immediately before seeding to give approximately 1×10^6 CFU/cm^3 planting mix. In the first experiment, the spores were suspended in phosphate buffered saline with 0.01% Tween-20, and in

the second experiment, the spores were suspended in OM broth with 0.01% Tween-20. Five imbibed seeds were planted in each container, approximately 0.5 cm deep, the planting mixture was covered with a plastic lid to maintain humidity, and containers were placed in a growth chamber with a 16 h light cycle at 21 °C. After 5 days, the lids were removed and each plant was inoculated with approximately 1×10^4 CFU *S. meliloti* strain USDA 105F21 in phosphate buffered saline. Additional nutrient solution was added to the bottom reservoirs as needed. Plants were removed 4 weeks after planting. Fresh weight of each plant was measured and the number of nodules on each plant counted. For each *Streptomyces–Sinorhizobium* combination, five containers were evaluated and each plant was analyzed as a subsample. Control treatments of each *Streptomyces* strain alone or *S. meliloti* alone were included in each experiment.

2.7. Statistical analyses

The Student's *t*-test was used to test for differences in plant biomass, nodulation, and disease severity between inoculated and non-inoculated treatments. Percent defoliation of each plant was converted to the arcsin value before analysis. To determine the influence of different strains on in vitro antibiosis, analysis of variance was performed using PROC GLM of SAS (SAS Institute, 1988). Fisher's unprotected least significant difference (LSD) test was used for mean comparisons. The Pearson correlation coefficient was used to evaluate the strength of the relationships between antibiosis against *P. medicaginis* and *S. meliloti* by the *Streptomyces* strains.

3. Results

3.1. Colonization of alfalfa seedlings by Streptomyces strains

Three *Streptomyces* strains, 93, GS6-17 and GS43-11, colonized roots, leaves, and nodules of alfalfa plants in the growth chamber assay (Fig. 1). The results from the two experiments were similar; data from one experiment are shown in Fig. 1. Streptomycete colonies recovered from inoculated plants had the morphology of the strain used for inoculation. Strain 93

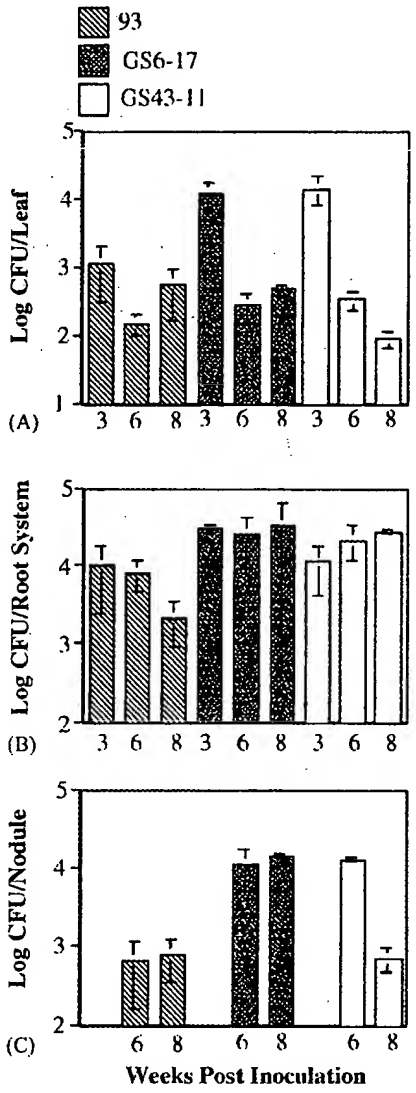

Fig. 1. Colonization of alfalfa plants by three *Streptomyces* strains: (A) mean log CFU per leaf; (B) mean log CFU per root; (C) mean log CFU per nodule. Each bar is the mean of five plants sampled at each time point with the standard deviation indicated.

had lower initial colonization densities than strains GS6-17 and GS43-11 on all organs. For all strains, colonization of the oldest trifoliolate leaf decreased significantly between 3 and 6 weeks after inoculation and colonization density remained low at 8 weeks after inoculation (Fig. 1A). The mean CFU recovered from individual root systems decreased over time on plants inoculated with strain 93 but remained constant on root systems inoculated with strains GS6-17

and GS42-11 over the 8-week period (Fig. 1B). Colonization of the upper 5 cm of each root system was approximately 10-fold greater (CFU/g root) than the remaining root system at all time points for all strains (data not shown).

The three *Streptomyces* strains were effective colonizers of alfalfa nodules. At 6 and 8 weeks after inoculation the mean CFU per nodule recovered from plants inoculated with strains 93 and GS6-17 were similar (Fig. 1C). From plants inoculated with strain GS43-11, the CFU per nodule decreased significantly from a mean of 1300 CFU per nodule at 6 weeks to 710 CFU per nodule at 8 weeks. There were no significant differences in nodule number or total nodule weight between 6 and 8 weeks for plants inoculated with strain GS43-11 (data not shown).

In one experiment, *Streptomyces* colonies with the morphology of the test strains were recovered from control plants indicating aerial movement of strains. At 6 weeks after inoculation, 20 and 38 CFU were recovered from the trifoliolate of two control plants, respectively. At 8 weeks after inoculation, *Streptomyces* colonies were recovered from the trifoliolate (8, 10, and 20 CFU per trifoliolate, respectively) and upper root system ($1–3 \times 10^3$ CFU per root system) of three control plants. No significant differences in shoot, root or nodule weight were observed between control and *Streptomyces*-inoculated plants.

Scanning electron microscopy confirmed the colonization of alfalfa roots by *Streptomyces*. In contrast to non-inoculated plants (Fig. 2A), a web of streptomycete filaments was observed along the entire root system of *Streptomyces*-inoculated plants (Fig. 2B) and many filaments appeared to be forming spore chains (Fig. 2C).

3.2. *In vitro antibiosis*

Initial double agar layer antibiosis assays with *P. medicaginis* 866 and *S. meliloti* 105F21 were conducted to test whether culture of multiple *Streptomyces* strains on an agar plate would influence the zone of growth inhibition by individual *Streptomyces* strains. Diameters of the zones of growth inhibition were similar for each specific *Streptomyces-P. medicaginis* or *Streptomyces–S. meliloti* combination, regardless of whether the *Streptomyces* strain was cultured alone or with four strains per plate (data not shown). Therefore,

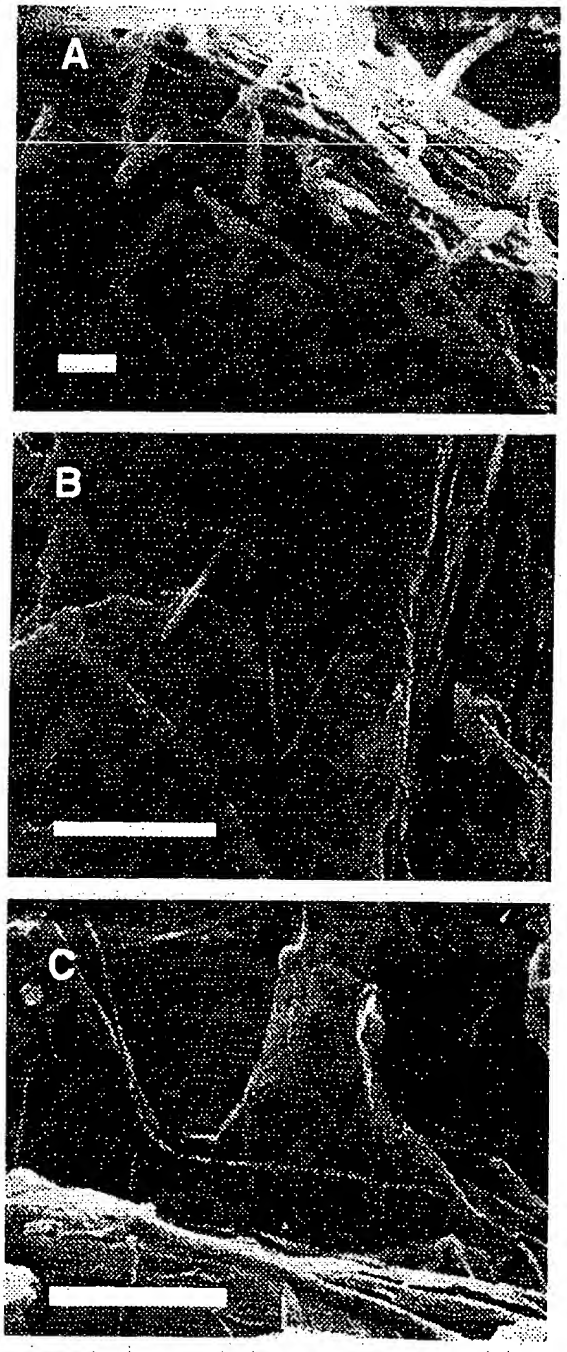

Fig. 2. Scanning electron micrographs of alfalfa roots inoculated with *Streptomyces* strain 93 at planting: (A) non-inoculated root. Bar = 50 μm; (B) *Streptomyces* filaments on inoculated root. Bar = 20 μm; (C) *Streptomyces* spore chains on inoculated root. Bar = 20 μm.

Table 1
Diameters (cm) of zones of in vitro growth inhibition of three strains of *Phoma medicaginis* by *Streptomyces* strains[a]

Streptomyces strain	*Phoma medicaginis*		
	866	T430	NY001
GS2-14	1.59 a	1.54 a	1.83 a
GS2-21	1.59 a	1.38 abc	1.63 abcd
GS2-17	1.59 a	1.35 abc	1.84 a
93	1.55 ab	1.19 bcd	1.39 cd
GS2-11	1.54 ab	1.51 a	1.73 ab
GS4-21	1.52 ab	1.43 ab	1.62 abcd
GS10-16	1.49 ab	1.01 d	0.68 e
GS6-17	1.45 ab	1.36 abc	1.59 abcd
GS43-5	1.35 ab	1.47 ab	1.7 abc
GS8-16	1.33 ab	1.28 abcd	1.43 bcd
GS8-22	1.26 ab	1.11 cd	1.38 d
GS8-1	1.20 b	1.15 cd	1.32 d
GS43-11	0 c	0 e	0 f
GS43-12	0 c	0 e	0 f

[a] Values followed by the same letter within a column are not significantly different (LSD, $P < 0.05$).

antibiosis assays testing sensitivity of 3 strains of *P. medicaginis* and 11 strains of *S. meliloti* to the *Streptomyces* strains were carried out using 4 *Streptomyces* strains per plate.

Growth of each *P. medicaginis* strain was inhibited by 12 of the 14 *Streptomyces* strains tested. There were no significant differences (Pearson's $P < 0.0001$) among the three *P. medicaginis* strains in their response to *Streptomyces*, although diameters of zones of inhibition varied among *P. medicaginis* strains (Table 1). Among the *Streptomyces* strains inhibiting growth, the strains with the greatest inhibitory activity towards *P. medicaginis*, based on the combined average diameters of the zones of inhibition, were GS2-11, GS2-14 and GS2-17. The strains with the least inhibitory activity were GS8-1 and GS8-22. Strain GS43-11 and GS43-12 had no inhibitory activity.

The zones of growth inhibition for *S. meliloti* were much smaller than those for *P. medicaginis* and fewer *Streptomyces* strains had inhibitory activity against *S. meliloti* strains (Table 2). Seven *Streptomyces* strains had no inhibitory activity against any *S. meliloti* strain or had only weak inhibitory activity against *S. meliloti* USDA1005. Two strains caused small growth inhibition zones on several *S. meliloti* strains while six strains inhibited growth of all or most *S. meliloti* strains. Based on combined sizes of zones of inhibition for all *S. meliloti* strains, *Streptomyces* strains GS2-11 and GS2-17 had the greatest inhibitory activity towards *S. meliloti*. The *S. meliloti* strains varied substantially in sensitivity to antibiotic inhibition; the most sensitive strains, based on number of inhibitory interactions, were USDA1005, USDA105F21, and USDA1093 and the most resistant strain was USDA1179.

A comparison of in vitro growth inhibition of *P. medicaginis* and *S. meliloti* by the *Streptomyces* strains tested shows that growth of each *P. medicaginis* strain and *S. meliloti* strain was inhibited by at least one *Streptomyces* strain (Tables 1 and 2). However, two *Streptomyces* strains (GS43-11 and GS43-12) were unable to inhibit the in vitro growth of any strain of either *P. medicaginis* or *S. meliloti*. Finally, there was no significant correlation among *Streptomyces* strains in growth inhibition of *P. medicaginis* and *S. meliloti* (Pearson's R value $= 0.458$, $P = 0.1$). Although some *Streptomyces* strains inhibited growth of both *P. medicaginis* and *S. meliloti* (GS2-11, GS2-17), other strains inhibited the pathogen, but had little to no effect on growth of the mutualistic symbiont (93, GS8-16). Thus, inhibition of the pathogen by a *Streptomyces* strain was not predictive of inhibition of the mutualistic symbiont.

3.3. Effect of Streptomyces strains on leaf spot symptoms

Six *Streptomyces* strains that had similar inhibitory activity against *P. medicaginis* 866 were tested for their effect on leaf spot symptoms in a detached leaf assay. After 5–6 days of incubation in a moist chamber, control leaves treated with only OM broth had a nominal amount of leaf yellowing and no tar spots (Table 3). Leaves treated with OM broth followed by *P. medicaginis* showed a high density of leaf spots and approximately 50% of leaf area was yellowed. Treatment of leaves with *Streptomyces* strain GS6-17 prior to pathogen inoculation caused a significant reduction in disease scores in both experiments compared with the treatment with *P. medicaginis* alone although disease symptoms were still evident. The other strains (GS8-16, GS2-11, 93, GS43-5, and GS2-17), although as inhibitory as GS6-17 against *P. medicaginis* strain 866 in antibiosis assays, did not significantly reduce disease symptoms.

Table 2
Diameter (cm) of zones of in vitro growth inhibition of *Sinorhizobium meliloti* by *Streptomyces* strains

S. meliloti	Streptomyces strains														
	GS2-11[a]	GS2-17	GS43-6	GS2-21	GS6-17	GS2-14	GS4-21	GS43-5	93	GS8-1	GS8-22	GS8-16	GS10-16	GS43-11	GS43-12
105F21	1.27 bc	1.23 abc	0.7 cd	0.78 bcd	0.74 bc	0.15 de	0.33 ab	0.44 ab	0	0	0	0	0	0	0
1005	1.45 b	1.43 a	1.28 ab	0.9 abc	1.18 ab	0.52 c	0.57 a	0.57 ab	0.17 a	0.07 a	0.03 a	0	0	0	0
1021a	1.48 b	1.35 ab	1.1 abc	0.97 ab	1.07 ab	0.15 de	0.23 bc	0 c	0	0	0	0	0	0	0
1031	2.03 a	1.32 ab	1.42 a	1.37 a	1.42 a	0 d	0 c	0.67 a	0	0	0	0	0	0	0
1035	1.45 b	0.97 bc	0.8 bcd	0.92 abc	0.83 bc	0 d	0 c	0 c	0	0	0	0	0	0	0
1045	1.47 b	1.05 abc	1.08 abc	0 e	0.73 bc	0 d	0 c	0.37 ab	0	0	0	0	0	0	0
1093	0.95 cd	0.9 bcd	0.7 cd	0.32 de	0.46 cde	0.27 cde	0.1 bc	0.23 bc	0	0	0	0	0	0	0
1098	0.69 d	0.45 d	0.39 d	0.08 e	0.24 de	0 d	0 c	0 c	0	0	0	0	0	0	0
1171	0.8 d	0.86 cd	0.61 cd	0.54 bcde	0.57 cd	0.29 cd	0.06 c	0 c	0	0	0	0	0	0	0
1179	1.08 cd	1.17 abc	0.72 cd	0.75 bcd	0 e	0 c	0 c	0 c	0	0	0	0	0	0	0
1180	0.95 cd	0.82 cd	0.68 cd	0.48 cde	0.23 de	0.28 d	0 c	0 c	0	0	0	0	0	0	0

[a] Values within a column followed by the same letter are not significantly different (LSD, $P < 0.05$).

Table 3
Effect of *Streptomyces* strains on leaf spot symptoms caused by *Phoma medicaginis*[a]

Inoculum	Mean disease score[b]	
	Experiment 1	Experiment 2
GS8-16	2.75 a	2.03 abc
P. medicaginis control	2.02 b	2.55 ab
GS2-11	1.91 b	2.67 a
93	1.84 b	1.69 bc
GS43-5	1.44 bc	2.19 abc
GS2-17	1.31 bc	1.84 abc
GS6-17	0.91 cd	1.53 c
Control[c]	0.44 d	0.38 d

[a] Numbers followed by the same letter within a column are not significantly different (LSD, $P < 0.05$).
[b] Values are the mean of the disease score of 12 leaves. 0 = no symptoms; 0.5 = 1–10 "tar spots" on each leaflet, no yellowing; 1 = 10–15 spots on each leaflet, approximately 25% of leaf yellow, 2 = more than 15 spots on each leaflet, approximately 50% of leaf yellow; 3=75% of leaf yellow, 4 = 100% of leaf yellow.
[c] Leaves were treated only with oatmeal broth.

Three *Streptomyces* strains were tested for their effect on defoliation caused by *P. medicaginis* using 8-week-old plants. The percent defoliation was greatest in both experiments in the non-inoculated control treatment (Table 4). In the first experiment, there was no significant effect of *Streptomyces* inoculation on defoliation. However, inoculation with strains GS43-11 and 93 at the time of planting significantly reduced defoliation in the second experiment.

Table 4
Effect of *Streptomyces* inoculation on defoliation by *Phoma medicaginis*[a]

Inoculum	Percent defoliation	
	Experiment 1	Experiment 2
Control	57 ab	59 a
93	61 a	41 b
GS43-11	56 ab	41 b
GS6-17	51 b	52 a

[a] Numbers followed by the same letter within a column are not significantly different (LSD, $P < 0.05$).

3.4. Effect of Streptomyces strains on nodulation

Six *Streptomyces* strains with a range of in vitro antibiotic activity against *S. meliloti* USDA105F21 were tested to determine their effect on nodulation and plant weight. In the first experiment, the planting mixture was inoculated with spores of the *Streptomyces* strains suspended in phosphate buffered saline. Neither the number of nodules per plant nor plant dry weight were significantly influenced by *Streptomyces* treatments compared to the treatment with *S. meliloti* alone (Table 5). In the second experiment, spores were suspended in OM broth. In this experiment, the number of nodules per plant varied significantly among *Streptomyces* treatments, although no treatment resulted in a significant difference in nodule number from the *S. meliloti* control. Nonetheless, plant weights were reduced significantly when plants were inoculated with GS2-11 and GS2-17 compared to inoculation with

Table 5
Effect of *Streptomyces* strains on nodulation and plant dry weight[a]

Inoculum	Nodule number per plant		Dry weight per plant (g)	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
GS43-6	18.6 a	24.3 a	0.42 a	0.53 b
GS6-17	17.3 ab	15.3 bc	0.39 a	0.52 b
GS8-16	16.6 ab	21.9 ab	0.38 a	0.50 b
S. meliloti control	16.3 ab	20.8 abc	0.43 a	0.62 ab
93	15.5 ab	23.6 a	0.44 a	0.76 a
GS2-17	15.0 ab	14.7 bc	0.41 a	0.26 c
GS2-11	14.5 b	16.4 ac	0.42 a	0.24 c
Control[b]	0 c	0 d	0.10 b	0.10 d

[a] Numbers followed by the same letter within a column are not significantly different (LSD, $P < 0.05$).
[b] Planting mixture treated only with phosphate buffered saline (Experiment 1) or oatmeal broth (Experiment 2).

S. meliloti alone (Table 5). These two strains showed the greatest in vitro growth inhibition against *S. meliloti*. However, other strains with strong in vitro antibiosis activity, GS43-6 and GS6-17, did not affect plant weights significantly.

4. Discussion

Streptomycetes are typically found in soils, but they have shown some potential for biological control of foliar pathogens (Gyenis, 2000). We found that alfalfa leaves, roots, and nodules were colonized by *Streptomyces* following inoculation of the planting mixture. Filaments as well as putative spore chains were observed along the entire root system of inoculated plants indicating active growth rather than passive movement of inoculum with plant growth. Relatively high population densities of *Streptomyces* occurred on alfalfa leaves up to 8 weeks after planting, when leaves had begun to senesce. Older alfalfa leaves are more susceptible to spring blackstem and leaf spot than young leaves, therefore maintaining effective population densities of *Streptomyces* as leaves age would be important for controlling this disease. Specific strains of *Streptomyces* have been found to effectively control foliar pathogens of *Poa pratensis* (Hodges et al., 1993) and hybrid poplar (Gyenis, 2000). Control of *P. medicaginis* with *Streptomyces* is attractive because the fungus attacks foliage, crowns, and roots of alfalfa plants (Leath et al., 1988) and inoculation of soil may be adequate to provide protection both above and below ground. Further experiments are needed to establish the longevity of introduced *Streptomyces* strains on alfalfa leaves and in the rhizosphere under field conditions. All *Streptomyces* strains tested attained relatively high population densities on alfalfa nodules, including those strains with antibiosis activity in vitro. Apparently alfalfa plants, or the rhizosphere soil of the plants, provide sufficient nutrients to support development of relatively high population densities of streptomycetes without affecting plant biomass accumulation. Nonetheless, streptomycete population densities on plant surfaces were affected by inoculum concentration. Preliminary experiments showed that a lower inoculum concentration (5×10^4 CFU/cm^3 of planting mix) resulted in lower population densities on alfalfa leaves and roots than a higher (10^6 CFU/cm^3) inoculum concentration. Thus, while the SEM study showed streptomycete growth on alfalfa root surfaces, some of the CFUs recovered from plants growing in ammended soil may arise from the original inoculum.

Diffusible chemicals from *Streptomyces* cultures have been shown to trigger antibiotic production by pathogen-suppressive *Streptomyces diastatochromogenes* PonSSII (Becker et al., 1997). Such interactions among streptomycetes may influence the antibiotic inhibition of pathogen strains in vitro. We tested whether culturing multiple *Streptomyces* strains in double agar layer antibiosis assays would affect the size of zones of growth inhibition against *P. medicaginis* or *S. meliloti*. Because the sizes of zones of inhibition were similar with single or multiple strains per plate, diffusible or volatile chemicals did not appear to impact in vitro antibiosis assays when test cultures were grown more than 20 mm apart. However, growth of *Streptomyces* strains in a closed atmosphere or at a closer distance to each other than that used here may influence the results.

The majority of the *Streptomyces* strains tested produced compounds highly inhibitory against growth of all three strains of *P. medicaginis* in vitro. The three strains, representing a range in geographic and genetic diversity within the species, responded differently to the panel of *Streptomyces* strains tested. This suggests that individual *Streptomyces* strains are producing distinct antifungal compounds, or different amounts of inhibitory compounds, and that pathogens vary in their sensitivity to these compounds. However, growth inhibition in culture did not correlate directly with control of disease symptoms. In the detached leaf assay, strain GS6-17 significantly decreased leaf spot symptoms caused by *P. medicaginis* on alfalfa leaves compared to the treatment with *P. medicaginis* alone (Table 3). However, in a defoliation assay, strain GS6-17 did not significantly decrease disease symptoms (Table 4). Inoculation of plants with strain GS43-11, which had no antibiotic activity against *P. medicaginis*, significantly decreased defoliation in one experiment. It is possible that antifungal compounds produced by the *Streptomyces* strains may not be produced on leaves or may not be effective once the fungus has penetrated the leaf surface. Furthermore, higher streptomycete population densities may be needed to have a significant effect on

disease control. Additional experiments are required to determine the optimal population density for effective control, to determine the competitive abilities of streptomycete strains against fungal pathogens and timing of antifungal compound production on the leaf surface. These experiments, and others using the same *Streptomyces* strains (Schottel et al., 2001; Xiao et al., 2002), indicate that antibiotic assays alone have a limited capacity to predict whether specific strains will be effective biological control agents for alfalfa diseases.

Overall, the *Streptomyces* strains had modest inhibitory effects on in vitro growth of the collection of *S. meliloti*. However, several strains affected growth of all *S. meliloti* strains to some degree. To determine if *Streptomyces* strains would affect nodulation, strains with different in vitro activities were allowed to colonize alfalfa seedlings for 5 days before inoculating with *S. meliloti*. Strains GS2-11 and GS2-17 showed marked in vitro activity against *S. meliloti*, however neither strain reduced nodule number significantly in either experiment compared to the treatment with *S. meliloti* alone. Interestingly, both strains significantly reduced plant dry weight in the second experiment, suggesting that the number of *S. meliloti* per nodule or their metabolic activity may have been reduced, leading to diminished nitrogen fixation. It is also possible that the *Streptomyces* strains produced plant growth-inhibiting compounds. Similar experiments were carried out with biological control strains of *Pseudomonas fluorescens* CHA0 that inhibit in vitro growth of *S. meliloti* due to production of pyoluteorin (Niemann et al., 1997). Over-production of pyoluteorin did not affect nodulation, however, lack of plant growth promotion by the over-producing strain was observed. In field soil, because of the presence of numerous different strains of *S. meliloti* and the variation in inhibition among *S. meliloti* strains, it is unlikely that inoculation with *Streptomyces* will significantly reduce either nodulation or plant dry matter accumulation.

These results lend further support to the concept that streptomycetes have the potential to contribute significantly to an integrated disease management system that includes alfalfa and other crops such as potato, corn, and soybeans due to their ability to colonize plants and decrease damage from a broad range of pathogens.

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Use of *Bacillus subtilis* as biocontrol agent. V. Biological control of diseases on maize and sunflowers

Die Verwendung von *Bacillus subtilis* als Mittel für den biologischen Pflanzenschutz. V. Biologische Bekämpfung von Krankheiten an Mais und Sonnenblumen

G. Schmiedeknecht[1], I. Issoufou[2], H. Junge[3], H. Bochow[2]

[1] Sächsische Landesanstalt für Landwirtschaft, Fachbereich Integrierter Pflanzenschutz, Stübelallee 2, D-01307 Dresden, Germany

[2] Humboldt-Universität Berlin, Landwirtschaftlich-Gärtnerische Fakultät, Fachgebiet Phytomedizin, Lentzeallee 55/57, D-14195 Berlin, Germany

[3] FZB Biotechnik GmbH, Glienicker Weg 185, D-12489 Berlin, Germany

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Summary

The effect of different environmental conditions on plant growth-promoting and antifungal activities of two different *Bacillus subtilis* strains were studied. For this purpose, the bacteria isolated and commercialized by FZB Biotechnik GmbH Berlin were tested for their *in vitro* and *in vivo* performances. First, the strains were screened for their antagonistic abilities against phytopathogenic fungi of maize and sunflowers. It was found that antifungal activities varied according to the composition of the assay medium, pH and temperature value. Low iron availability in the assay medium increased bacterial competition for this element and resulted further in a higher antifungal activity. Further, it was found that different nitrogen sources affected differently growth and spore production by *B. subtilis* strains. Higher growth and spore production rates were observed when the bacteria were incubated in Landy medium or in nitrate-nitrogen rather than in ammonium-nitrogen broth. Then the efficacy of the bacteria to promote plant health and growth was evaluated. These studies were conducted in culture solution and in soil under different ecological factors. The results suggest that these bacterial strains may produce substances that enhance plant growth and yield on maize and sunflowers. However, the results also showed that *in vitro* antibiotic activity did not always correlate with disease reduction on maize in greenhouse and field trials. Only in the case of the biological control of *Sclerotinia sclerotiorum* on sunflowers in field trials, a satisfying effectiveness was achieved. The results are discussed with regard to the general effect of plant growth and health-promoting substances produced by *Bacillus subtilis*.

Key words: *Bacillus subtilis; Fusarium oxysporum; Sclerotinia sclerotiorum;* biological control; plant growth promotion; nitrogen; maize; sunflowers

Zusammenfassung

An zwei verschiedenen *Bacillus subtilis* Stämmen wurde der Effekt unterschiedlicher Umweltbedingungen auf die pflanzenwachstumsfördernde und antifungale Aktivität untersucht. Die beiden von der FZB Biotechnik GmbH Berlin isolierten und kommerzialisierten Stämme wurden dazu auf ihre *in vitro* und *in vivo* Leistungen geprüft. Zuerst wurden die Stämme auf ihre antagonistischen Fähigkeiten gegenüber pflanzenpathogenen Pilzen bei Mais und Sonnenblumen getestet. Es wurde festgestellt, dass die antifungalen Aktivitäten variieren in Abhängigkeit von der Zusammensetzung des Testmediums, des pH-Wertes und der Temperatur. Geringe Verfügbarkeit von Eisen im Testmedium verbessert die

Konkurrenzfähigkeit der Bakterien und führt zu höherer antifungaler Aktivität. Außerdem wurde beobachtet, dass verschiedene Stickstoffquellen das Wachstum und die Sporulation der *B. subtilis* Stämme unterschiedlich beeinflussen. So wurden verbesserte Wachstums- und Sporulationsraten erzielt, wenn die Bakterien in Landy-Medium oder in Nährlösungen mit Nitratstickstoff inkubiert wurden im Vergleich zu Nährlösungen die nur Ammoniumstickstoff enthielten. Anschließend wurde die Wirksamkeit der Bakterien zur Förderung des Pflanzenwachstums und der Pflanzengesundheit bewertet. Diese Untersuchungen wurden in Kulturlösungen sowie im Boden bei unterschiedlichen ökologischen Bedingungen durchgeführt. Die Ergebnisse zeigten, dass von den Bakterienstämmen Substanzen produziert werden, die das Pflanzenwachstum fördern und den Ertrag erhöhen. Aber die Ergebnisse zeigten auch, dass die antibiotische Aktivität *in vitro* nicht immer mit der Bekämpfungswirkung bei pilzlichen Krankheiten an Mais *in vivo* korrelierte. Nur im Freiland zur biologischen Kontrolle von *Sclerotinia sclerotiorum* bei Sonnenblumen wurde eine gute Wirksamkeit erreicht. Die Ergebnisse werden aus Sicht einer offensichtlich generellen Wirkung pflanzenwachstumsfördernder und gesundheitsfördernder Substanzen von *Bacillus subtilis* diskutiert.

Stichwörter: *Bacillus subtilis; Fusarium oxysporum; Sclerotinia sclerotiorum;* biologische Bekämpfung; Pflanzenwachstumsförderung; Stickstoff; Mais; Sonnenblumen

1 Introduction

Biological control of soil-borne plant diseases and plant growth promotion by an application of specific micro-organisms to seeds or planting material has been studied extensively over the last years on different crops (Weller and Thomashow 1993; Koch 1996; Buchenauer 1998; Kilian et al. 2000; Bochow et al. 2001). Among these rhizobacteria, certain strains of *Bacillus subtilis* have been shown to suppress various plant diseases caused by soil-borne pathogens (Turner and Backman 1986; Mahaffee and Backman 1993; Backman 1995; Kim et al. 1997; Kilian et al. 1997; Schmiedeknecht et al. 1998; Steiner 1998). Currently, there is increasing interest in the introduction of bacterial biocontrol agents for managing soil-borne pathogens, partly as a response to public concerns about non-target effects of synthetic fungicides, but also because of a lack of effective control for soil-borne pathogens (Cook 1993). On the other hand, the complexity of the soil ecosystem makes biological control by introduced bacteria particularly challenging (Weller 1988). Under field conditions, however, most biological agents, including *Bacillus spp.*, are too variable in their performance to be used successfully as a common practice in agriculture and horticulture. Many factors contribute to the inconsistent performance of seed-applied bacteria given the complex interactions among host, pathogen, biological agent, and the environment (Pierson and Weller 1994; Raaijmaker et al. 1995). Major factors include the variability in root colonization by introduced bacteria and the expression of the genes involved in disease suppression and plant growth promotion (Weller and Thomashow 1994). Thus, population sizes vary from root to root, and some roots may be completely unprotected (Zimmer et al. 1998). Bull et al. (1991) reported that an inverse relationship between the population size of *Pseudomonas fluorescens* strain 2–79 on wheat roots and the number of take-all lesions indicates that incomplete colonization can reduce the potential for biological control by introduced bacteria. Another important factor that can contribute to inconsistent performance is variable production or *in situ* inactivation of bacterial metabolites responsible for disease control and plant growth promotion (Weller and Thomashow 1993). Normally, production of these metabolites *in vitro* depends only on cultural conditions. But *in situ* production is likely to be even more sensitive to the physical and chemical environment in soil and in the rhizosphere. Understanding the factors, which influence the performance of biocontrol of soil-borne plant pathogens is a great deal to the eventual improvement and a wide use of biocontrol methods. Several works put in a prominent position the ability of micro- and macro-elements to manage soil-borne diseases. Therefore, nitrogen has been intensively studied in relation to host nutrition and disease severity because of its requirement for plant growth (Henis and Chet 1968; Huber and Watson 1974; Smiley 1978; Barna et al. 1983; Wüthrich et al. 1991; Ownley et al. 1992; Engelkes et al. 1997). Ownley et al. (1991) reported that soil factors, e. g., ammonium nitrogen, sulfate sulfur, zinc, etc., were directly correlated with the biocontrol activity of

Pseudomonas fluorescens against take-all disease. Disease reduction is most often attributed to the improved nutrition that stimulates host defenses or to direct inhibition of pathogen growth, survival, germination and virulence (Duffy and Défago 1997). However, nitrogen sources and levels may activate indigenous population of micro-organisms which improve plant growth, but antagonistic to pathogens. While effects of temperature, pH value, organic matter content, and organic amendments on biocontrol agents have been reported, the influence of mineral amendments such as nitrogen for optimizing biocontrol has received little attention (Duffy and Défago 1997). It was the aim of this study to examine the effects of different nitrogen sources and rates on biocontrol activity of *Bacillus subtilis* strains, influenced by other ecological factors, on maize and sunflowers against different soil-borne diseases. An understanding of these effects would allow for more appropriate formulation and improved use recommendations for *Bacillus subtilis* and could lead to more consistent field performance with this beneficial micro-organism.

2 Material and methods

2.1 Culture solution studies

Maize seeds *(Zea mays L.)* cv. 'Felix' were surface-sterilized for 30 min in 3 % NaClO, washed in sterile distilled water and placed on Standard Nutrient Agar I (SNA, Merck) for 3 days at 27 °C. After 3 days, aseptic seedlings were transferred to sterile tubes (15 × 30 mm) containing about 19 ml of nutrient solution with the following composition: 3.5 g/l of KNO_3; 1.5 g/l of $NaNO_3$; 3.5 g/l of Na_2HPO_4; 5.25 g/l of KH_2PO_4; 0.5 g/l of KCl; 0.75 g/l of $MgSO_4{*}7H_2O$; 0.02 g/l of Fe - chelate; 0.001 g/l of $Na_2MoO_4{*}2H_2O$; 0.002 g/l of $CuSO_4{*}5H_2O$; 0.018 g/l of $MnSO_4{*}H_2O$; 0.002 g/l of $ZnSO_4{*}7H_2O$ and 0.023 g/l of $Na_2B_4O_2$. Sodium borate and iron chelate were sterile filtered and added to the solution after autoclaving (Kroffczyk et al. 1984). Tubes were covered with aluminium foil in order to avoid exposure of the roots to light. The seedlings were supported just above the solution by a Nescofilm screen. Trials were treated with a 1-day-old ammonium or nitrate fermentation broth in the form of cell-free filtrates or aqueous suspension of *Bacillus subtilis* cells containing about 1×10^8 cfu/ml. Three days later, the test system was inoculated with 0.1 ml of *Fusarium oxysporum* spore suspension containing about 1.1×10^5 cfu/ml. The pathogen was preliminary grown on potato dextrose agar (PDA, Merck) in the dark at 27 °C for 14 days, and then spores were washed from agar surface with sterile distilled water. Water losses during the growing period were compensated for with sterile distilled water. For trials where water consumption was more pronounced, nutrient solution was given up to the level (water) where its consumption was less. The plants were grown under growth chamber conditions at 16-h light and temperatures of 30 °C on the day and 20 °C in the night for 14 days. Five replicates of each treatment were arranged in completely randomized design. At the end of the experiment, the numbers of cfu, disease index and plant vitality were recorded. The study was repeated at different periods three times.

In order to establish a link between *in vivo* and *in vitro* activities of the different *Bacillus subtilis* strains, 10 ml of PDA or malt extract agar (MEA, Merck) mixed with 1 ml aqueous suspension of *B. subtilis* cells, cell-free culture filtrates, or with fermentation broth plus bacterial cells were plated on Petri dishes. After the media were solidified set, 5 ml of agar (Merck) were spotted on it. The dishes were then incubated in the dark at 27 °C for 3 days. After that time, 10 ml of PDA or MEA were again plated and seeded with an 8 mm plug of *Fusarium oxysporum, Sclerotinia sclerotiorum* or *Rhizoctonia solani* from 14-day-old cultures. After 7 days of incubation under different environment conditions, fungal growth was recorded.

2.2 *In vivo* activity of *Bacillus subtilis* strains in soil

Trials were conducted either in a growth chamber or in a greenhouse. Surface-sterilized maize seeds were sown in a 13-cm diameter plastic pot (two seeds/pot) containing a mixture of field soil and commercial soil (1 : 1 v/v), 350 g per pot. Field soil was obtained from Blumberg (Humboldt-University, Agricultural Experimental Station, Brandenburg, Germany). Soil was treated with water-dispersible formulation of *Bacillus subtilis* strains at 3×10^9 cfu/ml (*B. subtilis* FZB24 or FZB37

produced by FZB Biotechnik GmbH, Berlin, Germany). After 5 days, half of the pots were inoculated additionally with spore suspension of *Fusarium oxysporum* containing about 5×10^5 cfu/ml (see above). The other pots were not inoculated, but the natural soil inoculum potential of *F. oxysporum* was used. Each pot was amended with 230 mg/100 g soil of KNO_3 and Hoaglands nutrient. The composition of nutrient solution is as follows: 23 g/l of KNO_3; 1 g/l of KH_2PO_4; 0.5 g/l of KCl; 0.75 g/l of $MgSO_4{}^*7H_2O$; 1 g/l of H_3BO_4; 0.2 g/l of $ZnSO_4{}^*7H_2O$; 0.2 g/l of $Co(N0_3)_2{}^*6H_2O$; 1 g/l of EDTA; 1.5 g/l of $MnSO_4{}^*H_2O$; 0.2 g/l of $CuSO_4{}^*5H_2O$ and Na_2MoO_4. Six replicates per treatment were arranged in a completely randomized design. The study was repeated to prove statistically the obtained results. Trials were conducted either in a greenhouse or in a growth chamber. Four weeks after sowing, plant height, shoot and root fresh weight and relative disease levels were assessed.

2.3 Field evaluation of *Bacillus subtilis* on maize and sunflowers

Field trials on maize and sunflowers to evaluate efficacy of strain *Bacillus subtilis* FZB24 were conducted from 1995–1998 at the Humboldt-University, Agricultural Experimental Station in Blumberg. The experimental design was a randomized complete block with four replications. The experiments were carried out on a Pleistocene loamy-sand soil. This soil was low in nitrogen, potassium, phosphorus, and organic matter with a pH of 5.1 to 5.9. Each plot consisted of eight rows, each 15 m long, with 75 cm row-spacing. Maize cv. 'Elita' and sunflowers cv. 'Frankasol' were used. The biological treatment consisted of a water-dispersible granule formulation (maize starch as carrier) from strain *Bacillus subtilis* FZB24 (1×10^{11} cfu/g). Further, a chemical treatment with a fungicide (thiram) (commercial coated seeds) and an untreated control were used for comparison. The applications of *Bacillus subtilis* were carried out before planting as a dipping treatment (1×10^9 cfu/ml). Normal cultivation practices for maize and sunflowers were used including chemical weed control. During and at the end of the growing season, the disease incidence and the plant growth parameters were recorded. After disease evaluation, the maize plants of each plot were harvested and the yields were determined.

3 Results

3.1 *In vitro* and *in vivo* biocontrol activity of *Bacillus subtilis* strains in culture solution

Previously, we studied the ability of *Bacillus subtilis* strains to inhibit fungal growth *in vitro*. First, *Bacillus subtilis* isolates were screened for antagonistic activity against phytopathogenic fungi of maize and sunflowers (Tab. 1). In *in vitro* plate assays, mycelial growth of these fungi was strongly restricted by the two tested strains of *Bacillus subtilis* (FZB24 and FZB37). The strain *B. subtilis* FZB24 produced larger inhibition zone and therefore was used in field investigations. Furthermore, the *in vitro* plate assays indicated that the size of inhibition depended on the media, the pH-value and the temperature. The antagonistic activity of the bacteria increased when the temperature was increased, especially against *Sclerotinia sclerotiorum* and *Rhizoctonia solani*. On the other hand, against *Fusarium oxysporum*, this temperature effect could not be proved. The highest activity of the bacteria was recorded when the

Table 1. *In vitro* inhibition effects of culture filtrates (1 ml) or cell spore suspensions of *Bacillus subtilis* isolates on fungal growth

Tab. 1. *In vitro* Hemmwirkungen von Kulturfiltraten und Sporensuspensionen von *B. subtilis* auf das Pilzwachstum

	Application of culture filtrates (Average fungal growth %)	Application of spores suspensions (Average fungal growth %)
Check[1]	100,0	100,0
Fusarium oxysporum	78,9	34,4
Sclerotinia sclerotiorum	61,1	33,4

[1] Without some *Bacillus subtilis* treatments.

media was weakly acid (pH 5.7). In neutral and weakly alkaline media, the different *Bacillus subtilis* strains have shown activity only against *Sclerotinia sclerotiorum*. The supplementation of Fe^{+3} ions to the assay medium led to a variation in antagonistic activities of *Bacillus subtilis* strains. Thus, a significant increase in antifungal activities of *Bacillus subtilis* FZB24 and FZB37 was observed when the concentration of iron in the medium was increased by 0.5 mg/l. However, further increases in iron concentration resulted in a decrease of bacterial activity.

The aims of the further *in vivo* studies were to compare the biocontrol and plant growth promotion abilities of two interesting *Bacillus subtilis* strains from *in vivo* testing under standard conditions, and also to investigate the effects of ammonium and nitrate nitrogen on those activities. According to the strains *B. subtilis* FZB24 and FZB37 used and the origin of the cell's fermentation media, a variable biocontrol activity was observed (Fig. 1).

Results from these *in vivo* tube tests indicated that strain *Bacillus subtilis* FZB24 developed better activities against *Fusarium oxysporum* when grown in nitrate broth. The average disease reduction of this tested strain amounted significantly to 88,4 % compared to results obtained when the cells were incubated in ammonium broth (30,2 %). On the other hand, the strain *B. subtilis* FZB37 showed relatively better performance against *Fusarium oxysporum* when grown in ammonium broth, but no effects in nitrate broth. Generally, biocontrol activity of the two strains FZB24 and FZB37 was identical when the strains were preliminary grown in ammonium (30,2 %). Furthermore, these results indicated that globally disease severity was more reduced when maize plants were treated with *Bacillus subtilis* FZB24 than with FZB37.

In prolonged observation, plant growth promotion was also detected and this depended on the bacterial strain and the nitrogen source used to incubate the bacteria. The studied maize plants showed better growth parameters in the test tubes when treated with *B. subtilis* isolate FZB24 cells incubated in nitrate broth, than when incubated in ammonium broth (Tab. 2). For instance, after inoculation with

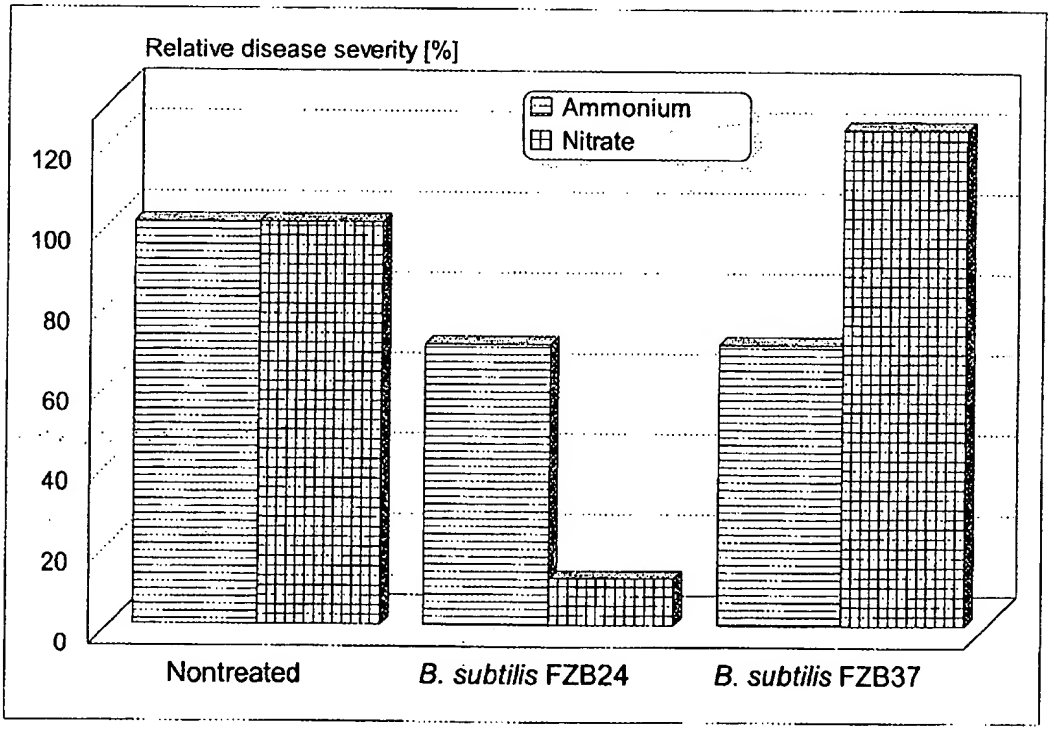

Fig. 1. Biocontrol activity of different *Bacillus subtilis* strains after special precultivation against *Fusarium oxysporum* on maize in culture solution in growth chamber test tubes trials 1998.
Abb. 1. Antagonistische Wirkungen verschiedener *B. subtilis*-Stämme nach unterschiedlicher Vorkultivierung gegenüber *F. oxysporum* an Mais in Kulturlösung im Pflanzenwuchsschrank 1998.

Table 2. Plant growth promotion effects of different *Bacillus subtilis* strains after special precultivation in culture solution and *Fusarium oxysporum* inoculation on maize in growth chamber test tubes trials 1998

Tab. 2. Wirkungen verschiedener *B. subtilis*-Stämme auf das Pflanzenwachstum von Mais nach unterschiedlicher Vorkultivierung und Inokulation mit *F. oxysporum* in Kulturlösung im Pflanzenwuchsschrank 1998

	Nitrate[1]		Ammonium[1]	
	Shoot fresh weight (g)	Root fresh weight (g)	Shoot fresh weight (g)	Root fresh weight (g)
Check[2]	2,05	1,25 a	2,05	1,25 a
B. subtilis FZB24	2,50	1,90 b	2,10	1,40 ac
B. subtilis FZB37	2,30	1,40 a	2,10	1,60 bc
	n.s.		n.s	

(1) Cells were preliminary incubated in ammonium or nitrate broth for 24 h.
(2) Without some *Bacillus subtilis* treatment.
Values with different letters are statistically significant according to Duncan's Multiple Range Test ($p \leq 0.05$).

F. oxysporum, a significant increase of average root fresh weight by 52 % was obtained when plants in the test tubes were treated with nitrate fermentation broth of *B. subtilis* FZB24. Otherwise, root growth was increased significantly (about 28 %) when treated with cells of *Bacillus subtilis* FZB37 incubated in ammonium broth. Generally, the test tube trials showed that strain *B. subtilis* FZB37 exhibited superior effects on plant growth promotion on maize when grown in ammonium broth than strain *B. subtilis* FZB24.

3.2 *In vivo* activity of *Bacillus subtilis* strains under growth chamber conditions

Growth chamber experiments in a mixed soil (commercial and field soils 1 : 1 v/v) showed a similar biocontrol activity by the two *Bacillus* strains when a dried spore formulation was used for soil treatment in comparison to the *in vivo* tube tests. So, the disease incidence of *Fusarium oxysporum* was reduced significantly up to 50 % after application of *Bacillus subtilis* FZB24 or FZB37 in comparison to the untreated control. Under these standardized conditions, plant growth parameters were promoted by the two bacterial strains, plant height and shoot and root fresh weight were significantly increased (Tab. 3). Differences were also detected in ability of different bacteria to promote plant growth. However, *B. subtilis* FZB37 exhibited better plant growth parameters. So, soil treatment with this strain caused more than 100 % shoot fresh weight and more than 180 % root fresh weight in comparison to the untreated control. On the other hand, plant height of maize was better promoted (41 %) when soil was treated with *B. subtilis* FZB24 than with FZB37 (25 %). Generally, in growth chamber trials in natural soil, the bacterized maize plants grew taller, shoots and leaves appeared greener and more vigorous than untreated plants.

Table 3. Plant growth promotion effects on maize by different *Bacillus subtilis* strains under natural infection conditions of soil in growth chamber trials 1998

Tab. 3. Wirkungen verschiedener *B. subtilis*-Stämme auf das Pflanzenwachstum von Mais bei natürlichem Infektionsdruck des Bodens im Pflanzenwuchsschrank 1998

	Plant height (mm)	Shoot fresh weight (g)	Root fresh weight (g)
Untreated Control	369 a	3,9 a	1,3 a
B. subtilis FZB24	521 b	7,2 b	2,8 b
B. subtilis FZB37	462 b	8,1 b	3,7 b

Values with different letters are statistically significant according to Duncan's Multiple Range Test ($p \leq 0.05$).

3.3 *In vivo* activity of *Bacillus subtilis* strains in greenhouse

The effectiveness of two tested strains of *Bacillus subtilis* was also demonstrated in three greenhouse trials. These investigations, carried out on maize, showed a variable disease reduction according to the studied bacteria isolate and the pathogen inoculum. However, the biocontrol activity of *B. subtilis* isolate FZB24 in natural infested soil was low. On the other hand, the biological treatment was significantly superior to untreated control when the soil was additionally inoculated with the pathogen *Fusarium oxysporum* (Fig. 2). In this case, the average disease severity was reduced up to 85 %. In contrast, treatment with the isolate *B. subtilis* FZB37 was completely ineffective in naturally infested soil. Still more, the average disease severity was increased by 23 % in comparison to the untreated trials. Nevertheless, biocontrol activities of strain FZB37 were observed exclusively in an artificially infested soil. The average disease severity decreased by 65 % in comparison to the untreated control, but less when plants or soil were treated with *B. subtilis* FZB24.

Furthermore, our greenhouse trials demonstrated that special cultivation methods of *Bacillus subtilis* strains exhibit different effects on plant biomass development. However, Table 4 shows that *Bacillus subtilis* FZB24 increased plant growth parameters by all the three tested states but more significantly when nitrate fermentation broth or the commercial formulations were used for soil treatment. In that case, plant height was increased more than 25 % and 15 %, respectively, concerning the shoot- or root fresh weight, it was increased only between 2 % and 8 %. At the opposite, despite an ineffective plant height promotion and decreased root fresh weight, soil treatment with ammonium fermentation broth considerably increased shoot fresh weight. The same observation was made in the case of *Bacillus subtilis* FZB37.

3.4 Field evaluation of *Bacillus subtilis*

Results from field experiments at the Blumberg site from 1995–1998 on sunflowers showed that the strain *Bacillus subtilis* FZB24 significantly decreased the occurrence of *Sclerotinia sclerotiorum* com-

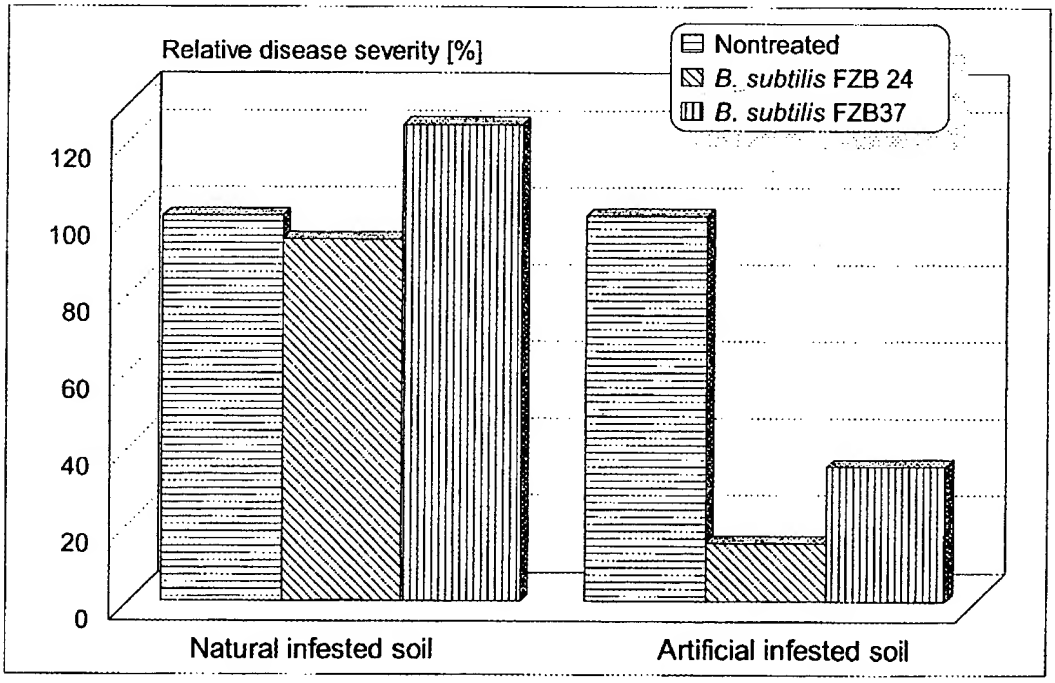

Fig. 2. Biocontrol activity of different *Bacillus subtilis* strains against *Fusarium oxysporum* on maize at different infection pressure in greenhouse trials, 1998 (n = 3 trials).
Abb. 2. Antagonistische Wirkungen verschiedener *B. subtilis*-Stämme bei unterschiedlichem Infektionsdruck gegenüber *F. oxysporum* bei Mais im Gewächshaus 1998 (n = 3 Versuche).

Table 4. Plant growth promotion effects on maize of different *Bacillus subtilis* strains after special precultivation in greenhouse trials 1998 (n = 3 trials)

Tab. 4. Wirkungen verschiedener *B. subtilis*-Stämme nach unterschiedlicher Vorkultivierung auf das Pflanzenwachstum von Mais im Gewächshaus (n = 3 Versuche)

	Bacillus subtilis FZB24			*Bacillus subtilis* FZB37		
	Plant height (mm)	Shoot fresh weight (g)	Root fresh weight (g)	Plant height (mm)	Shoot fresh weight (g)	Root fresh weight (g)
Check	467 a	14,0 a	4,4	467 ac	14,0 a	4,4
Nitrate[1]	586 b	15,0 a	4,8	510 bc	15,5 a	4,0
Ammonium[1]	480 ac	18,3 b	3,8	408 a	18,7 b	4,3
KNO_3[2]	545 bc	15,2 a	4,5	546 b	13,8 a	3,7
			n.s.			n.s.

[1] Cells were preliminary incubated in ammonium or nitrate broth for 24 h.
[2] Commercial KNO_3-formulation (FZB Biotechnik GmbH, Berlin) was used.
Values with different letters are statistically significant according to Duncan's Multiple Range Test ($p \leq 0.05$).

pared to the untreated control. Over the 4-year period, relative average disease severity of growing sunflower plants was declined by bacterization with this strain by 62.0 % (ranging from 58.3–66.1 %). The bacterial treatment showed similar control effects compared to chemical compound thiram which caused a relative reduction ranging from 62.5–72.4 %. Both the biological and chemical treatments were significantly more effective compared to the untreated control, but no significant differences could be detected among themselves. Contrary to the results obtained on sunflowers, field experiments from 1997–1998 on maize indicated that there are no direct effects of strain *Bacillus subtilis* FZB24 on the occurrence of soil-borne pathogens. However, indirect effects could be found. So, the germination of maize seeds was superior to untreated control (Tab. 5). The same effects of sprouting were also detected on sunflowers (Fig. 3). Generally, under field conditions, the bacterized maize and sunflower seeds germinated better and seedlings and plants grew also more vigorously than untreated ones. For example, the sunflowers had a larger basket diameter, a stronger and longer stalk. On maize over the 2-year period, both biological and chemical treatments showed increases of yields (dry weight) ranging between 17 % (thiram) and 16 % *(B. subtilis)* compared to the untreated control. But these plant growth promotion effects, however, were not statistically significant.

4 Discussion

Biological control of soil-borne plant pathogens and plant growth promotion by micro-organisms has gained widespread acceptance as a potential tool in optimizing agricultural productivity. Understanding the potential use of an antagonist for biological control of a disease depends on the answers to a

Table 5. Influence of bacterial and chemical treatments on plant growth of maize in field trials 1997–1998

Tab. 5. Einfluss chemischer und biologischer Behandlungen auf das Pflanzenwachstum bei Mais Freilandversuche 1997–1998

	Seed germination (plants/m²)	Fresh weight (dt/ha)	Dry weight (dt/ha)	Corn yield (dt/ha)
Untreated control	10,30	281,0	112,6	48,8
Chemical control	11,31	317,4	131,8	59,8
B. subtilis FZB24	11,24	295,2	130,9	59,0
	n.s.	n.s.	n.s.	n.s.

Values with different letters are statistically significant according to Duncan's Multiple Range Test ($p \leq 0.05$).

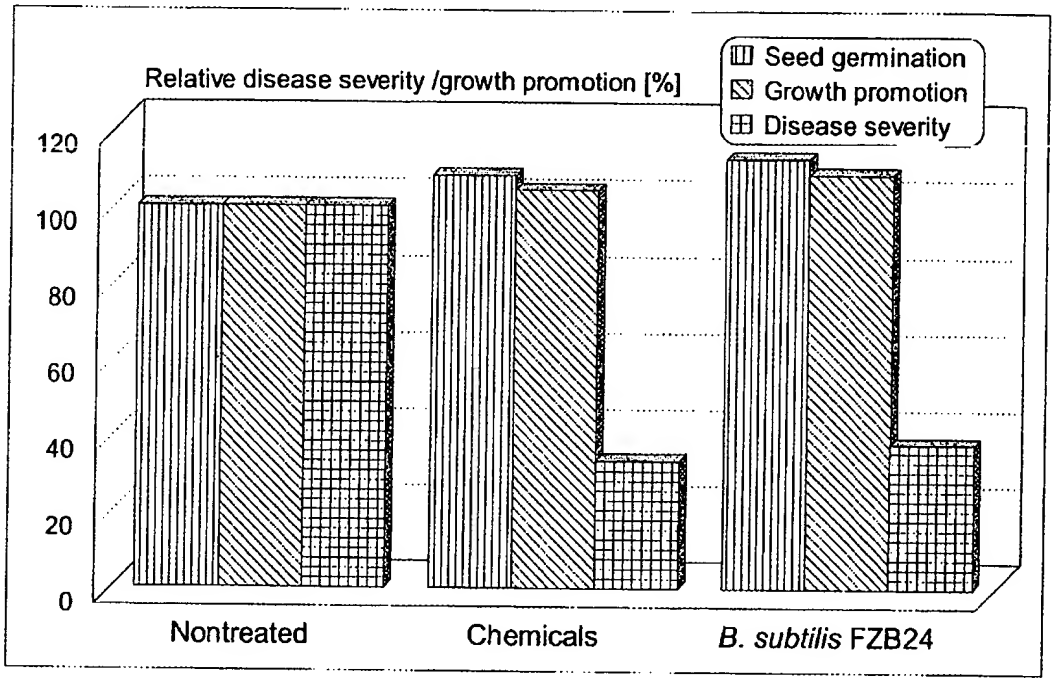

Fig. 3. Influence of bacterial and chemical treatments on the reduction of *Sclerotinia sclerotiorum* as well as on plant growth of sunflowers in field trials 1995–1998.
Abb. 3. Einfluss chemischer und biologischer Behandlungen auf den Befall mit *S. sclerotiorum* sowie auf das Pflanzenwachstum von Sonnenblumen, Freilandversuche 1995–1998.

series of questions regarding the interactions of the host (crop), pathogen, and the antagonist. Many micro-organisms are known to be producers of antibiotics or toxic metabolites that could inhibit strongly fungal growth *in vitro*. But antibiotic production on artificial media *in vitro* alone cannot provide sufficient proof of the involvement of antibiotics in biocontrol activity *in vivo*. Antibiotic production and activity by biocontrol strains was often demonstrated *in vitro*, however, and there is therefore no direct evidence that the same antibiotics are produced and responsible for disease control when biocontrol agents are applied to plant tissues or in plant rhizosphere (Leifert et al. 1995). On the other hand, antibiotic production may confer on micro-organism a selective advantage in competition for nutrients and spaces within their ecological niches. Consequently, the selection pressures in nature may promote populations of antibiotic producers, even though better survival opportunity exist in the environment of these organisms (Buchenauer 1998). However, many pathogenic fungi are sensitive to *Bacillus subtilis* cell suspension or its culture filtrates. Besides, many *Bacillus* strains are also known to suppress fungal growth *in vitro* by the production of one or more antifungal antibiotics, which were identified as peptide antibiotics (Krebs et al. 1998). Some of these antibiotic-producing strains were also shown to suppress fungal plant diseases *in vivo* (Fravel 1988; Bochow 1992). This fungal growth suppression by special strains of *Bacillus* appears as a complex of competition, parasitism, root colonization, and antibiosis. However, these strains produce irrespective of peptide antibiotics a range of other metabolites including biosurfactants, chitinase or other fungal cell wall-degrading enzymes, volatiles and compounds that elicit plant resistance mechanisms (Kehlenbeck et al. 1992; Fiddaman and Rossall 1994; Leifert et al. 1995; Dolej 1998). All these metabolites have also been implicated in biocontrol. Furthermore, *Bacillus* showed sufficient abilities to colonize roots of a wide range of culture plants (Liu and Sinclair 1992; Mahaffee and Backman 1993). Our results presented here suggest that bacterization of maize and sunflowers with *Bacillus subtilis* not only provides suppression of the pathogens, but also enhances crop growth and yield. On the one hand, this suggestion is supported by growth chamber and greenhouse data for significant reductions of *Fusarium oxysporum* in bacterized maize plants (Fig. 2) and by data of field evaluation for significant reduction of *Sclerotinia*

sclerotiorum in bacterized sunflower crops (Fig. 3). Biological control measures which employ *Bacillus subtilis* like those characterized and used in this study have been successful against important diseases caused by soil-borne fungi such as *Fusarium, Gaeumannomyces, Pythium, Rhizoctonia, Sclerotinia,* and others (Broadbent et al. 1971; Merriman et al. 1974; Weller 1988; Backman 1995; Bochow et al. 1996; Zhang et al. 1996; Kim et al. 1997; Schmiedeknecht et al. 1997, 1998). On the other hand, data presented here on enhancement of growth and yield of maize and sunflower plants treated with *Bacillus subtilis* reveal that these different strains have the potential to cause enhanced growth and yield of these crops for example by production of phytohormonally active metabolites (Dolej 1998; Bochow et al. 2001). Bacterized plants grew more vigorously and appeared greener than did those in non-bacterized greenhouse pots or field plots. At harvest, the bacterized maize plants were taller than the non-bacterized plants (Tables 3 and 4). Though a 5 % increase in fresh weight in bacterized crops is non-significant, perhaps much higher yields can be obtained with more efficient strains of *Bacillus subtilis* or more effective bacterization procedures.

Further, the current study demonstrates, in general, antagonism in *in vitro* plate assays or in solid culture media is not enough to qualify a rhizobacterium like *Bacillus* as potential agent for effective biocontrol, for distinct plant growth promotion or sufficient root colonization. Thus, despite the fact that the strain *Bacillus subtilis* FZB37 exhibited no effective evidence of antifungal activity by its culture filtrates in *in vitro* plate assays against *Fusarium oxysporum.* Nevertheless, plant or soil treatment with this strain induced excellent plant growth promotion also in presence of the pathogen, and this as well as the strain *B. subtilis* FZB24 that produced better antagonistic and antifungal (antibiotic) *in vitro* activity. Similar results with strain *B. subtilis* FZB37 were also achieved with potatoes (Schmiedeknecht et al. 1996). It appears further remarkable that plant growth promotion rates were in strong relationship on the bacteria cell states, i. e., the origin or the nature of the incubation broth. As a matter of fact, under greenhouse conditions, seedlings treated with bacterial fermentation broth from ammonium produced significantly more shoot fresh weight than those treated with nitrate fermentation broth or with the commercial KNO_3-formulation produced by FZB Biotechnik GmbH Berlin. Our findings let presume that, on the one hand, ammonium influenced the capability of *Bacillus subtilis* to enhance fresh weight of plants. On the other hand, nitrate affected the capability of *Bacillus subtilis* to promote plant height and biocontrol activity against *Fusarium oxysporum.* Both nitrate and ammonium seems to promote the activity of the tested *Bacillus subtilis* strains, because most of the bacterial cells are in an active stage, i. e., ready for root colonization. However, the data suggest that it may be possible to enhance the level of biocontrol or plant growth promotion through manipulation of nitrogen sources present during production of the bacteria. Concerning the effects of nitrogen in biocontrol, Henis and Chet (1968) pointed out the relationship between the decrease in germination of sclerotia of *Sclerotium rolfsii* incubated in soil amended with nitrogenous substances and the increase in number of micro-organisms associated with these sclerotia. The suggestion was that the efficiency of nitrogenous amendments could result in the production of antibiotics or toxic metabolites, which decrease fungal growth. Therefore, these amendments affect sclerotial germinability indirectly by increasing bacterial antagonistic activity. Engelkes et al. (1997) reported that the role of nutrient source can be critical to success of a biocontrol agent, and must be evaluated to ensure reasonable efficacy. Thus, the benefits of different bacterial fermentation or formulation techniques are illustrating the importance of ecological influences for the capability of bacteria to colonize plant roots and to promote activities of micro-organisms (Xi et al. 1996; Ownley and Clark 1998). However, the success of biocontrol may be greatly affected by different formulations and the formulation can also influence shelf life and survival of the biocontrol agents. It seems evident that *in vitro* fungal suppression may confer the susceptibility of bacteria for competition, roots colonization and antibiosis *in situ.* On the other hand, no one of these modes of action appears to be predominant, and so they may also act together to affect biocontrol and/or to promote plant growth. In a sense, Howie et al. (1986) pointed out that antibiotic production had a primary importance, while seed and root colonization were of secondary importance. Likewise, Kloepper and Schroth (1981) reported that mutants without antibiosis effects *in vitro* also do not promote plant growth. In contrast, it is evident through our *in vitro* studies on antibiosis that culture filtrates of our investigated *Bacillus subtilis* strains exhibited different activity against a wide range of pathogenic fungi according to the nature of growth, test media, temperature and pH-value. While culture filtrate of *B. subtilis* FZB37 *in vitro* failed to

inhibit fungal growth of *Fusarium oxysporum,* its spore's suspension exhibited a sufficient performance in sense of plant growth promotion and of antibiosis or competition for nutrients in presence of this particular fungi. We suppose that this strain *B. subtilis* FZB37 *in vivo* may produce compounds different from those produced *in vitro*, which induce or enhance plant growth *in situ*. In comparison, strain *B. subtilis* FZB24 exhibited a steady antibiosis against numerous fungi *in vitro* and *in vivo*. Further, this strain *B. subtilis* FZB24 also performed biocontrol activities and plant growth promotion on different plants through a number of studies conducted under different ecological and biotic conditions (Bochow 1992; Dolej and Bochow 1996; Kilian et al. 1998; Schmiedeknecht and Bochow 1998, Schmiedeknecht et al. 1998; Steiner 1998). Currently, we are conducting investigations to determine in details the required ecological and biotic conditions in order to improve biocontrol and plant growth promotion activities of *Bacillus subtilis*. This study has identified more effective strategies that enhance crop growth and yield as well as for biological control of maize and sunflower diseases using micro-organisms and should aid commercialization of biological agents like *Bacillus subtilis*. Further, our thoughts could play an important role as a component in biologically based management strategies in agriculture.

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JoAnne M. Rantz, Editor
Keith B. Lewis, Publishing Assistant
P.O. Box 700
Lockeford, California 95237-0700
Telephone (209) 727-3439
FAX (209) 727-5004

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Biological Control of Grape Crown Gall with Non-tumorigenic *Agrobacterium vitis* Strain F2/5

T. J. BURR[1]* and C. L. REID[2]

A non-tumorigenic strain (F2/5) of *Agrobacterium vitis* produces an agrocin that inhibited *in vitro* growth of 21 of 25 *A. vitis*, two of 10 *A. tumefaciens* biovar 1, and none of nine biovar 2 strains. It inhibited both tumorigenic and non-tumorigenic strains. When applied to wounds on potted woody grape trunks (*Vitis vinifera* L. cvs. Chardonnay and Riesling) in the greenhouse, the gall sizes were significantly reduced for seven of 10 *A. vitis*, one of two *A. tumefaciens* biovar 1 and one of one biovar 2 strains. The numbers of inoculation sites at which galls developed was reduced for all but one *A. vitis* strain. There is a good, but not perfect, correlation between *in vitro* sensitivity to the agrocin and control *in vivo*. Co-inoculation of F2/5 with pathogen was as effective or more effective in most cases than pre-inoculation of F2/5. When pathogen was inoculated prior to F2/5, the level of control was greatly reduced. Control was most effective when equal concentrations of F2/5 and pathogen were inoculated and declined for ratios of 1:10 and 1:100 (F2/5 to pathogen). F2/5 contains three plasmids, none of which hybridize with a probe, pTHE17, consisting of the T-DNA from *A. tumefaciens* strain C58.

KEY WORDS: *Agrobacterium vitis*, *A. tumefaciens*, *A. tumefaciens* biovar 3, crown gall, biological control

Agrobacterium vitis (16) formerly designated as *A. tumefaciens* biovar 3 or biotype 3, is the most predominant species causing crown gall disease on grape (3). The bacterium survives systemically in grape and is frequently disseminated in propagation material (4). Recently, methods have been tested for developing pathogen-free vines using shoot tip culture (6) and heat therapy (7). However, once clean vines are obtained, it will be necessary to protect them from reinfection from *A. vitis* inoculum that may persist in decaying grape debris in vineyard soils (1,3,5).

Biological control of crown gall has been highly successful on several crops using *A. radiobacter* strain K-84 (12); however, *A. vitis* is not controlled by this strain (3). Therefore, several laboratories have attempted to identify biological controls that are effective against *A. vitis* (13,17,19,20). Xiaoying and Wangnian (21) isolated a non-tumorigenic strain of biovar 1 (HLB-2) that inhibited growth of several *A. vitis* strains and suppressed development of the disease on grape in the greenhouse. Staphorst evaluated 16 strains including strain F2/5 which was effective against some *A. vitis* strains in laboratory and in greenhouse tests (18). The purpose of this research was to test F2/5 against several strains of *Agrobacterium* from different geographical regions. F2/5 was evaluated for *in vitro* activity and for control on grape in the greenhouse. Ratios of pathogen to F2/5 were tested as well as timing of application of F2/5 to in relation to inoculation with the pathogen. Plasmid content of F2/5 was compared to that of K-84 and HLB-2 and Southern analysis was done to determine if native plasmids of F2/5 hybridize with a DNA probe consisting of T-DNA from *A. tumefaciens* strain C58 (8).

Materials and Methods

Bacterial strains: Strains used are listed in Table 1. All strains were stored at -80°C in cryogenic storage medium (1.2 g nutrient broth, 22.5 g glycerol, 85 mL distilled water). Inocula for experiments were grown on potato dextrose agar (PDA, Difco) or MG medium (10). Strain F2/5, previously referred to as F2 (17), was kindly provided by Dr. J. L. Staphorst (Plant Protection Research Institute, Private Bag X14, Pretoria 0001, Rep. of South Africa) and obtained by APHIS permit No. 1 PPQ 584.

***In vitro* assay:** Evidence for the production of an agrocin was obtained by placing a 10 µL drop of a suspension of strain F2/5 containing about 10^8 colony-forming units (CFU) (optical density of 0.1 at 600 nm determined with spectrophotometer) was applied to the center surface of a 9-cm petri plate containing 20 mL of MG medium. Plates were incubated for 48 hours at 28°C, at which time bacterial cells were killed by chloroform vapor, and bacterial growth was scraped from the plates. Subsequently, the surface of the plates were sprayed until wetted with suspensions (about 10^8 CFU/mL) of bacteria to be tested for sensitivity. Forty-four *Agrobacterium* strains were tested for agrocin sensitivity including *A. tumefaciens* biovar 1 (5 tumorigenic, 6 non-tumorigenic), biovar 2 (5 tumorigenic and 5 non-tumorigenic), and *A. vitis* (17 tumorigenic and 6 non-tumorigenic). Plates were incubated at 28°C, and the sizes of inhibition zones were recorded after 48 hours.

[1]Department of Plant Pathology, New York State Agricultural Experiment Station, Cornell University Geneva, NY 14456.

*Author to whom correspondence should be addressed.

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Table 1. Sensitivity of *Agrobacterium vitis* and *A. tumefaciens* biovars 1 and 2 to agrocin produced by strain F2/5.

Strain	Source[a]	Biovar or species	Tumor[b]	Sens. to[c] agrocin F2/5
CG628	Burr, grape, NY	1	+	-
CG632	Burr, grape, NY	1	+	-
CG640	Burr, grape, NY	1	+	-
CG648	Burr, grape, NY	1	+	-
CG90	Burr, grape, NY	1	-	-
CG91	Burr, grape, NY	1	-	-
CG210	Burr, grape, NY	1	-	+
CG219	Burr, grape, NY	1	-	-
C58	Dickey, cherry, NY	1	-	++
HLB2	Xiaoying, hop, China	1	-	-
CG907	Burr, raspberry NY	2	+	-
A-4	Ark, rose, CA	2	+	-
K-47	Kerr, Australia	2	+	-
R-3	Dickey,rose, NY	2	+	-
CG978	Burr, pussy willow, NY	2	+	-
CG414	Burr, soil, NY	2	-	-
CG423	Burr, soil, NY	2	-	-
CG438	Burr, grape root, WA	2	-	-
K84	A. Kerr, peach, Australia	2	-	-
CG47	Burr, NY	Av[d]	+	++
CG49	Burr, NY	Av	+	+++
CG56	Burr, MI	Av	+	+
CG60	Burr, NY	Av	+	++++
CG78	Burr, NY	Av	+	++
CG98	Burr, VA	Av	+	++++
CG102	Burr, VA	Av	+	++++
CG106	Burr, MS	Av	+	+++
CG108	Burr, NM	Av	+	++++
CG660	Burr, NY	Av	+	-
CG693	Burr, NY	Av	+	+
CG696	Burr, NY	Av	+	++
Ag57-81	Panagopoulos, Crete	Av	+	++++
AA 25.	Ercolani, Afghanistan	Av	+	+++
1860 (3)	Bazzi, Italy	Av	+	+
NW-161	Bien, Germany	Av	+	++++
K306	Ophel, Australia	Av	+	++
CG472	Burr, WA	Av	-	++
CG481	Burr, NY	Av	-	-
CG482	Burr, WA	Av	-	++++
CG483	Burr, WA	Av	-	++++
CG487	Burr, WA	Av	-	-
CG488	Burr, WA	Av	-	++++
CG669	Burr, grape, NY	?[e]	-	-
CG670	Burr, grape, NY	?	-	+

[a]Author, plant from which isolated, and state or country.

[b]Tumorigenicity as determined on sunflower, kalanchoe, tomato, and/or grape.

[c]Relative size of inhibition zone (mm): - = no inhibition, $0 < + \leq 1$, $1 < ++ \leq 2$, $2 < +++ \leq 3$, $3 < ++++$.

[d]Av = *A. vitis*. All strains were isolated from grape.

Assay on grape: Rooted grape, *Vitis vinifera L.* cuttings (about one-month-old cvs. Chardonnay and Riesling), were used. They had been stored prior to planting as cuttings of dormant canes with three nodes. Inoculations with bacteria were made by applying 75 µL of bacterial cell suspensions in holes that were bored in the living woody stems of the plants with an electric drill. The drill bit diameter was about 6 mm and holes were drilled to the depth of the pith. Three or four inoculations were made to each plant and inoculation sites were wrapped with parafilm. The numbers of inoculation sites at which galls developed and gall size (mm^2 of gall surface area) were recorded 8 weeks after inoculation. Sterile distilled water was applied as a negative control. Data were analyzed using the Waller-Duncan K-ratio test.

Biological control activity of F2/5 against different strains of *A. tumefaciens* was tested. Wound sites were first inoculated with 75 µL of a suspension (about 10^8 CFU/mL) of F2/5 or sterile distilled water. About 90 minutes later, they were inoculated with the same volume and concentration of *A. vitis* or *A. tumefaciens* strains. To test the effect of applying the pathogen and F2/5 at the same time, a suspensions containing equal concentrations of F2/5 and pathogenic strains (10^8 CFU/mL for each) were inoculated to wound sites.

We also tested the effects of applying F2/5 at different times in relation to inoculation with the pathogen. Suspensions of F2/5 and *A. vitis* strain K306, of equal concentration (about 10^8 CFU/mL) were used. In one experiment, F2/5 or sterile distilled water were applied about 10 minutes, five hours, and 16 hours prior to inoculation with K306. In another experiment, strain K306 or sterile distilled water were applied to wound sites and F2/5 was applied about 10 minutes, three hours, six hours, or 18 hours later. All experiments were repeated at least once.

Biological control as affected by concentration of F2/5 in relation to concentration of the pathogen was tested using ratios of 1:1, 1:10, and 1:100 of F2/5 to pathogen. In these experiments, F2/5 was applied at a concentration of about 10^8 CFU/mL 90 minutes before inoculation with pathogen. *A. vitis* strains included CG106 and K306. Pathogens and F2/5 were also applied alone to plants as described above. Suspensions of pathogens and F2/5 were serially diluted and plated on PDA medium to verify populations and

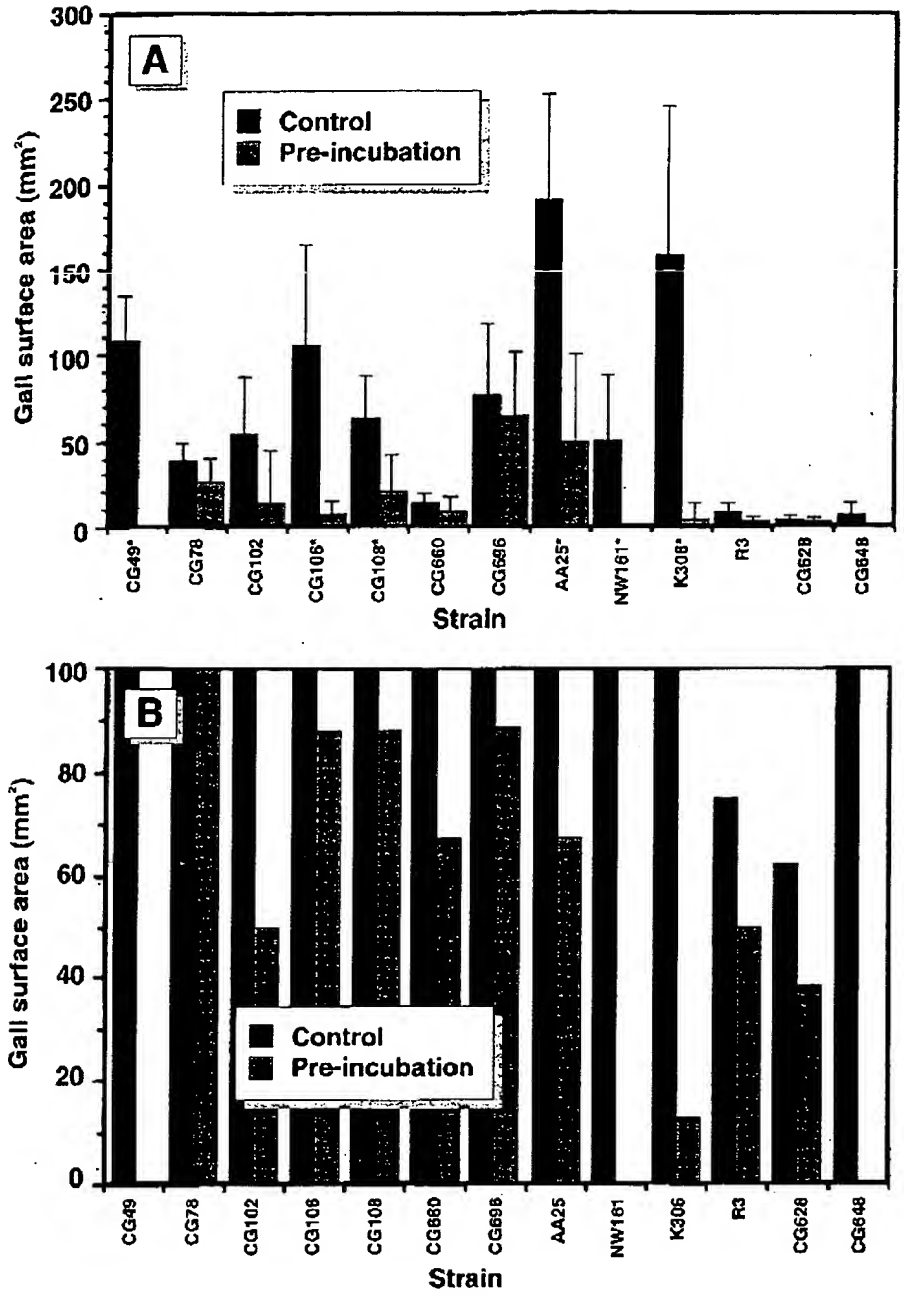

Fig. 1. (A) Effect of pre-inoculation of F2/5 on gall size. (B) The percentage of inoculation sites at which galls developed.

ratios. All experiments were repeated at least once.

Agrocin effect in mixed inoculations: The effect that secreted agrocin in F2/5 cultures may have on survival of cells of the pathogen that were co-mixed with F2/5 prior to inoculation was determined. Cultures of F2/5 and strains K306, CG49, and CG51 (spontaneous rifampicin mutant of CG49) were grown on PDA or PDA plus rifampicin (50 µg/mL) and then suspended in distilled water to a concentration of about 10^8 CFU/mL. F2/5 and single pathogenic strains were mixed at equal concentrations in distilled water and incubated at 25°C. Populations of pathogen and F2/5 were measured immediately after mixing and after one and four hours by plating on PDA (or PDA plus rif) and on RS medium (F2/5 only produces small colonies on RS that are distinct from strains CG49 and K306). Plates were incubated 48 hours (PDA) or six days (RS) at 28°C, and colonies were counted. The experiment was repeated once.

Southern analysis of F2/5 plasmids: Plasmid DNA was isolated from *Agrobacterium* strains K-84 , HLB-2 and F2/5, and CG56 using the method of Slota and Farrand (17). Undigested plasmids were electrophoresed in 0.7% agarose in TBE (14) at 5 V/cm. DNA was stained with ethidium bromide and visualized. DNA was then Southern transferred to GeneScreen Plus-Hybridization Transfer Membrane (Dupont, NEN Research Products) by alkaline transfer. Hybridizations were done with T-DNA probe, pTHE17 (8) which was labeled by random primed incorporation of digoxigenin-labeled deoxyuridine-triphosphate using a non-radioactive DNA labeling kit (Genius, Boehringer Mannheim, Indianapolis, IN).

Membranes were pre-hybridized at 68°C for 135 minutes and hybridized at 68°C for 18 hours in 0.03 mL hybridization solution/cm² membrane containing 25 ng/mL of labeled probe pTHE17. Following post-hybridization, rinses, blocking, and incubation with antibody-conjugate solution and subsequent rinses, the membrane was placed within a hybridization bag that was open on two sides and saturated with a sufficient quantity of LumiPhos 530 (Boehringer Mannheim, for chemiluminescent detection of alkaline phosphatase) to saturate the membrane. It was then incubated in the dark (wrapped in aluminum foil) for one minute. The excess LumiPhos 530 was then drained from the hybridization bag, the bag was sealed and wrapped in foil and incubated at 37°C for 30 minutes. The membrane was subsequently placed in a film cassette, exposed to X-ray film (Kodak XAR) for eight minutes, and then developed.

Results

***In vitro* assay:** F2/5 produces an agrocin that is primarily inhibitory to *A. vitis* strains (Table 1). However, two *A. tumefaciens* biovar 1 strains (CG210 and C58) were sensitive and three *A. vitis* strains (CG660, CG481, and CG487) were not. Sensitivity to agrocin was not correlated with tumorigenicity, since some

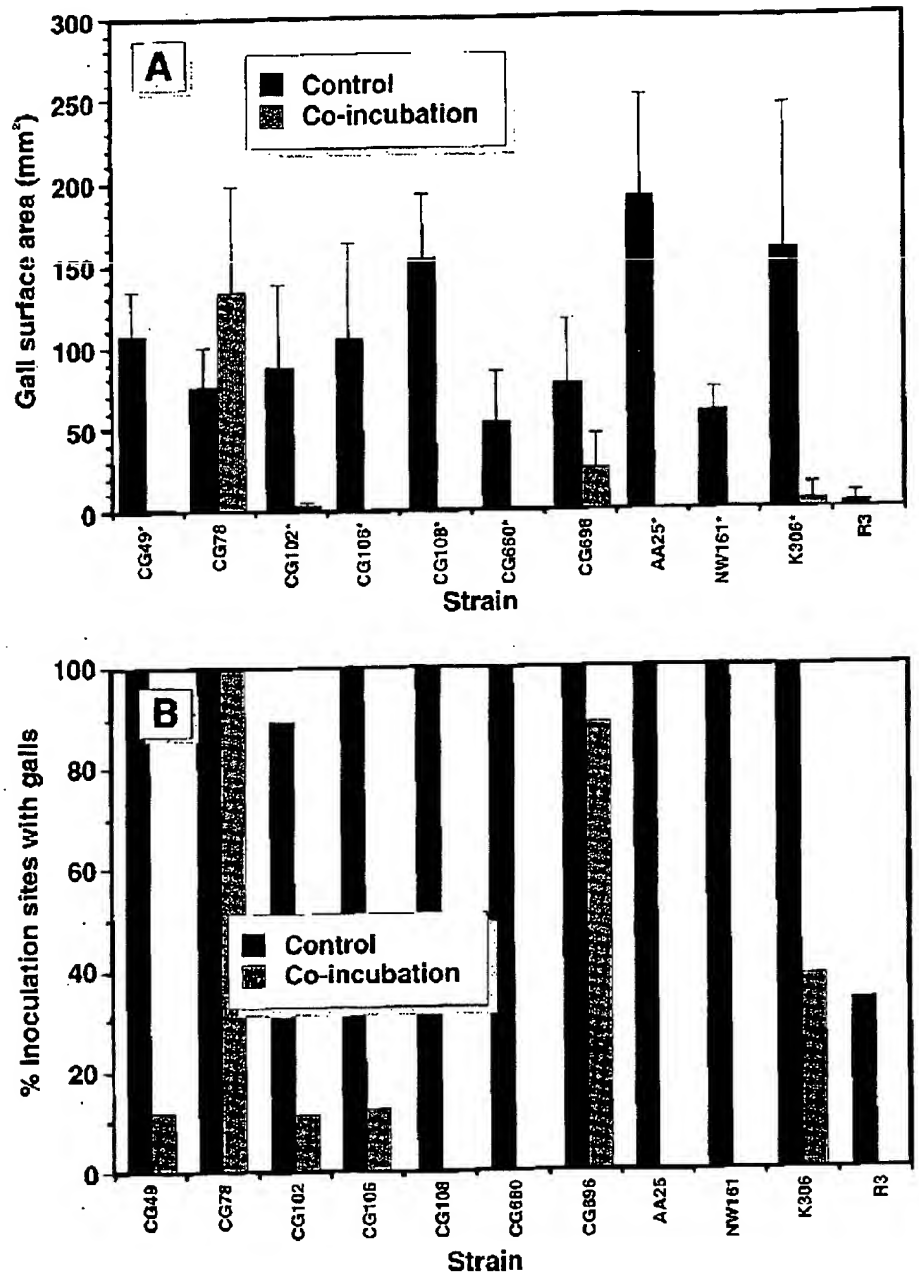

Fig. 2. (A) Effect of coinoculation of F2/5 and *Agrobacterium* strains on gall sizes. (B) The percentage of inoculation sites at which galls developed.

tumorigenic and non-tumorigenic strains of *A. vitis* were highly sensitive as determined by relative inhibition zone sizes that were produced on bioassay plates.

***In vivo* activity:** F2/5 significantly reduced gall size caused by seven of 10 *A. vitis*, 1 of 2 *A. tumefaciens* biovar 1 and 1 biovar 2 strains when applied prior to inoculation with the pathogen (Fig. 1A). It reduced the numbers of galls on plants inoculated with all strains except one, *A. vitis* (CG78) (Fig. 1B). When applied at the same time as the pathogen (co-inoculated), it significantly reduced gall sized and the number of galls produced for eight of nine *A. vitis* strains and one *A. tumefaciens* biovar 2 (Fig. 2A and 2B). Again F2/5 did not affect gall formation by CG78. In no case did F2/5 induce galls.

F2/5 was most effective when applied at the same concentration as the pathogen (Fig. 3). At a ratio of 1:10, (F2/5 to pathogen) galls sizes for strain CG106, but not for K306, were smaller than the control. At 1:100, gall sizes for both strains were not different from the control. The number of galls induced by CG106 was not greatly reduced when F2/5 was pre-inoculated at the 1:1 ratio, but was significantly reduced when CG106 was co-inoculated with F2/5.

F2/5 was most effective in reducing tumor size and number of inoculation sites with galls when applied prior to or at the same time as inoculation of the pathogen (Fig. 4). When the pathogen was applied prior to F2/5, gall size and numbers of inoculation sites with tumors were not different from plants that were only inoculated with the pathogen.

Agrocin effect in mixed inoculum: There was no detectable effect of residual agrocin from F2/5 on *A. vitis* survival when mixtures of the strains were made in water. The populations of F2/5 in the inoculum mixtures equaled 4.3×10^7 CFU/mL, whereas CG49, CG51, and K306 were 4.9×10^7, 1.5×10^7, and 2.5×10^7, respectively. After four hours in the mixture with F2/5. populations of CG49, CG51, and K306 were 7.7×10^7, 3.7×10^7, and 4.3×10^7, respectively.

Plasmid profiles and Southern analysis: Plasmid profiles of K-84, HLB-2, and CG56 were identical to those previously reported (8). Strain F2/5 contains three plasmids (Fig. 5A) one being smaller than typical plasmids associated with tumorigenic *Agrobacterium* (Ti-plasmids). Whereas a single plasmid from K-84, HLB-2, and CG56 hybridized with pTHE17, there was no hybridization with F2/5 (Fig. 5B).

Discussion

Several aspects that are important to the development of commercial usage of F2/5 have been determined. F2/5 is an *A. vitis* strain that reacts with a species-specific monoclonal antibody (2) and produces a secreted polygalacturonase (15) (Burr, unpublished data, 1992). It is non-tumorigenic, was originally isolated from grape (17), and therefore like other *A. vitis* strains, is likely to survive well epiphytically and endophytically on grape (1,3,4,5). It also produces an agrocin that is

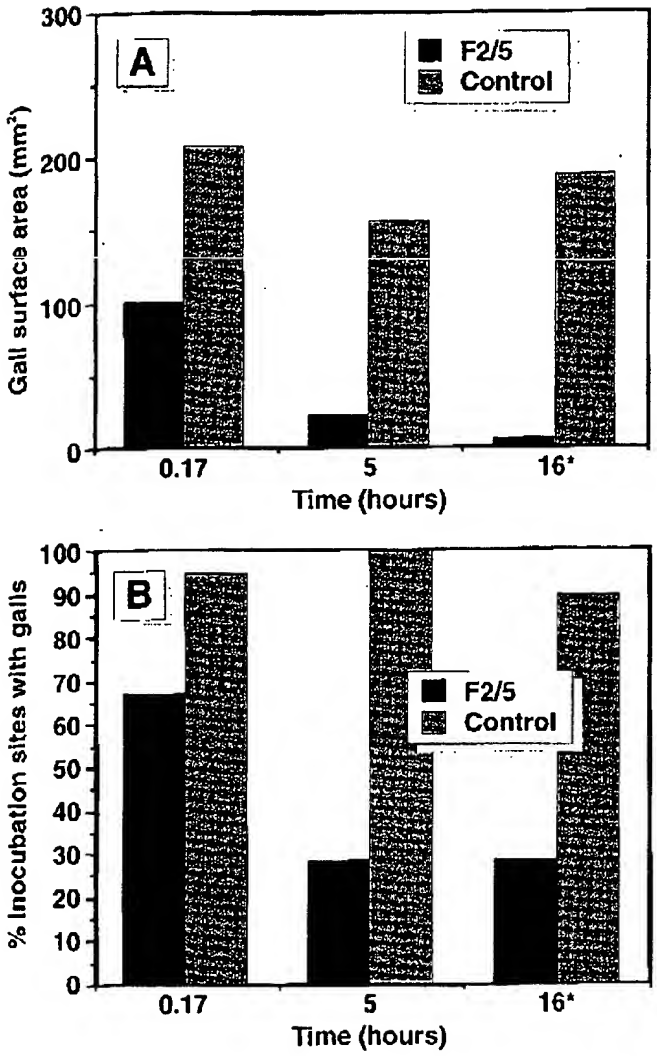

Fig. 3. (A) Effect of time of application of F2/5 in relation to inoculation with pathogen on gall size. (B) The percentage of inoculation sites at which galls develop.

primarily active against tumorigenic and non-tumorigenic *A. vitis* strains. In contrast, sensitivity to the agrocin produced by strain K-84 is encoded by determinants on certain Ti plasmids (tumorigenic strains), and *A. vitis* strains are not sensitive (3). We have demonstrated that F2/5 is active against several *A. vitis* and *A. tumefaciens* strains and that the effectiveness of control will depend on applying F2/5 prior to infection by the pathogen and at concentrations equal to or greater than that of the pathogen. It will be essential now to conduct field experiments by treating *A. vitis*-free vines prior to planting in pathogen-infested soils.

Other potential biological controls for grape crown gall have been reported from laboratories in South Africa (19) and China (12,20). One strain from China, HLB-2, was recently evaluated against a group of *A. vitis* strains from the U.S. (9). HLB-2 is a non-tumorigenic *A. tumefaciens* biovar 1 strain that produces an agrocin that is active against several strains of *Agrobacterium*. Although it reduces gall formation *in vivo*, it may not be as active as F2/5, since poor control was achieved when co-inoculations were made using a 1:1 (HLB-2:pathogen) inoculum ratio. Galls were prevented, however, for six of eight and seven of eight strains when 10:1 and 100:1 ratios were tested, respectively.

Although most strains of *A. vitis* are sensitive to the agrocin that is produced by strain F2/5, the role of the agrocin in control of crown gall on grape is not clear. For example, strain CG78 is sensitive to agrocin *in vitro*, but gall size or numbers of inoculation sites with galls are not significantly reduced. Another strain, CG660, is not sensitive to F2/5 *in vitro*, but gall size was reduced in the co-inoculation experiments and numbers of galls at inoculation sites were reduced. Similarly, the *A. tumefaciens* biovar 1 and 2 strains used for *in vivo* experiments were not sensitive to the F2/5 agrocin *in vitro*, but galls sizes were significantly reduced for CG648 (biovar 1) when F2/5 was pre-inoculated and for CG936 (biovar 2) in the co-inoculation experiment. Numbers of inoculations sites with tumors was also reduced for the biovar 1 and 2 strains. We, therefore, feel that the agrocin may be involved with biological control, but other mechanisms are likely to be involved and need further investigation. Staphorst, *et al.* (18) reported similar inconsistencies between *in vitro* sensitivity of pathogens to F2/5 and subsequent biological control. Similar conclusions have been drawn with strain K-84 in that agrocin is an important factor contributing to biological control; however, other factors such as competition for binding sites are also likely to be involved (10).

It is interesting that co-inoculation of F2/5 together with the pathogen is as effective and sometimes more effective than applying F2/5 to wound sites prior to the pathogen. We demonstrated that this is not due to killing of the pathogen in the inoculum mixture by residual agrocin. In fact, PDA medium that is used for growing F2/5 for biological control experiments produces low to non-detectable levels of agrocin as measured by plate bioassays (Burr, unpublished data, 1992). Therefore, we are not certain how F2/5 is suppressing gall formation by the pathogen. If agrocin is a primary factor, it appears that it must be produced at the wound site on the plant. It is also possible that F2/5 is competes for binding sites or affects tumorigenisis in some other way.

The genetic determinants for agrocin production by F2/5 have not been identified. The mechanism of agrocin F2/5 sensitivity is apparently different from that of K-84 (agrocin 84) which is Ti plasmid dependent, since non-tumorigenic strains of biovar 3 may be sensitive. Strain F2/5 carries three plasmids, two of which are comparable in size to the Ti plasmid (about 200 Kb) and none of which hybridize with T-DNA probe, pTHE17. In contrast, a 200 Kb plasmid from K-84, known to encode nopaline catabolism, shares some homology with pTHE17 (8). This result supports greenhouse pathogenicity tests that demonstrate F2/5 is non-tumorigenic.

Conclusions

We have demonstrated that *A. vitis* strain F2/5 has

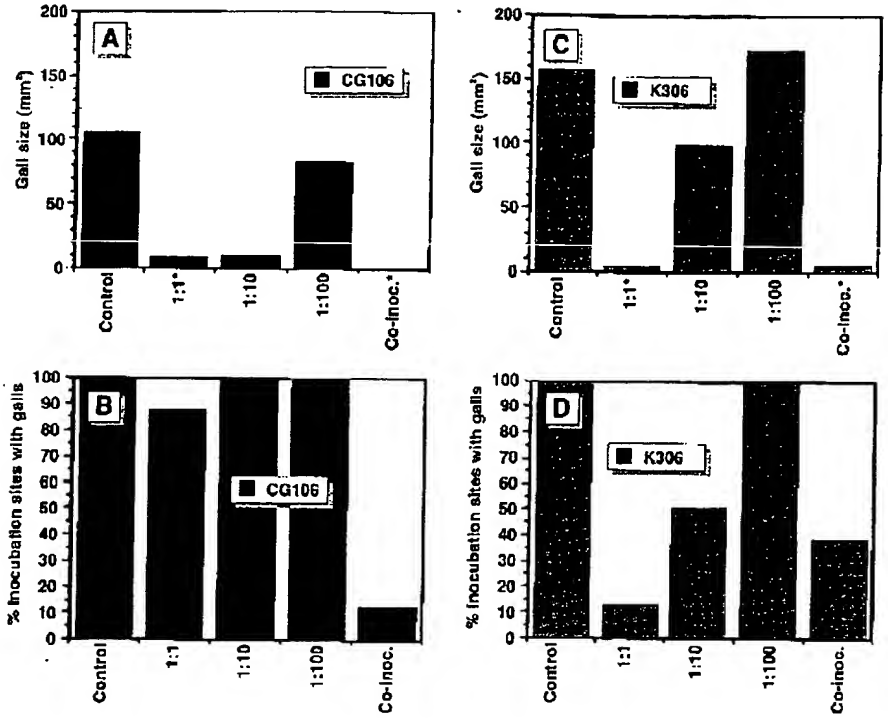

Fig. 4. (A and C) Effect of ratio of F2/5 to pathogen on galls size. (B and D) The percentage of inoculation sites at which galls develop.

potential as a biological control of grape crown gall. Because *A. vitis* is host-specific to grape, it is likely that F2/5 will survive and compete well on grape. Further research on the competitiveness of F2/5 in relation to tumorigenic *A. vitis* strains is necessary. F2/5 is clearly non-tumorigenic because of its failure to induce galls on plants in the greenhouse and to hybridize with a T-DNA probe. It will be essential to apply F2/5 to grapevines prior to infection and at a concentration equal to or greater than that of the pathogen. We propose that disease-free vines will be inoculated with F2/5 prior to planting in the vineyard. It will now be important to conduct field experiments with F2/5 by treating plants and growing them in *A. vitis*-infested soils. It would be best to conduct such trials in cold climates, where freeze injury predisposes plants to crown gall infection.

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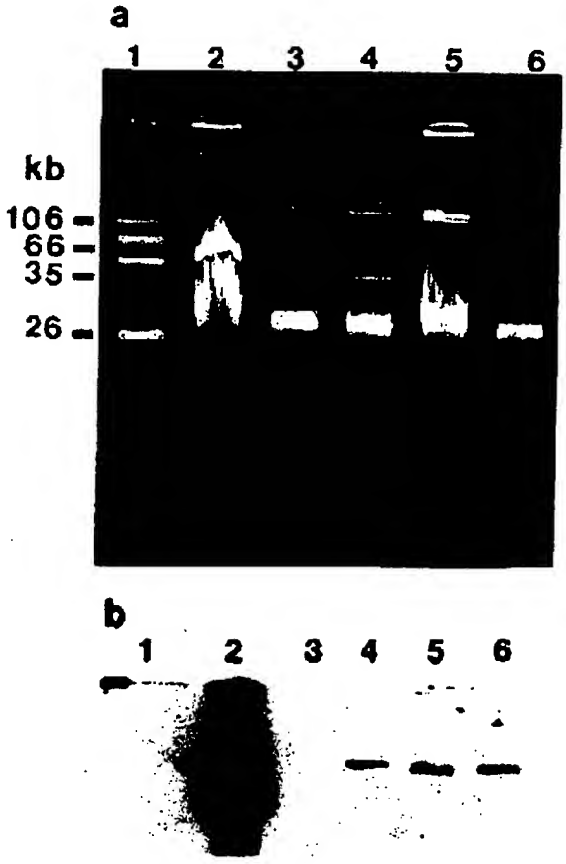

Fig. 5. (A) Plasmid profile. (B) Corresponding Southern analysis (hybidization with probe pTHE17) of strains F2/5 (lane 3), K84 (lane 4), HLB-2 (lane 5), and tumorigenic *A. vitis* strain CG56 (lane 6). Lane 1 is *Erwinia stewartii* strain SW2 (used as size marker, obtained from D. Coplin, Ohio State University) and lane 2 is *E. coli* strain DH-1 containing pTHE.

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